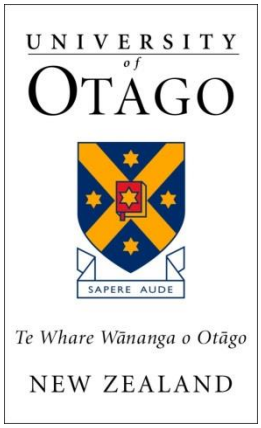

Integrated profiling, fingerprinting, and chemometrics as a tool for distinguishing the impact of Pulsed Electric Fields (PEF) pre-treatment, winemaking, and storage on Merlot grape juice and wines

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ABSTRACT

The thesis mainly aims to evaluate the impact of Pulsed Electric Fields treatment of high electric field strengths (33.1 and 41.5 kV/cm) with specific total energy levels of 16.47 and 49.40 kJ/L on Merlot grapes, PEF-processed at commercial scale (500 kg/hr). The Merlot juice obtained immediately after PEF processing was examined at pre-maceration (PM). The fermenting must and the resulting wine were assessed on the completion of maceration - alcoholic (MAF) and malolactic fermentations (MLF), respectively. The wine stability was further tested during bottle storage at different temperatures (4°C, 25°C, and 45°C) for 150, 120, and 56 days, respectively. Comprehensive assessment of these samples was achieved through combined targeted profiling of phenolic compounds, colour, and oenological properties, and untargeted volatile fingerprinting coupled with multivariate data analysis in order to confidently identify discriminant markers that are most affected by winemaking, storage, or PEF treatment. These markers were further linked to relevant (bio)-chemical reactions.

Upon winemaking, grape musts at PM typically contained the highest concentrations of C6 compounds (compounds composed of six carbons and other elements) which are green odourants. After MAF, more phenolic compounds and aroma precursors were extracted and transformed by yeast metabolism, which developed the colour and aroma of the fermenting musts. At the end of MLF, the extracted phenolic and aroma compounds decreased and transformed into the derivative compounds as a result of lactic acid bacteria metabolism. It was important to note that grape samples at each winemaking stage were consistently distinguished according to the intensity of specific energy applied before maceration. High specific energy PEF treatments of 49.40 kJ/L produced grape juice with the highest concentrations of anthocyanins, flavonols, stilbenes, hydroxycinnamic acids, flavanonol, and flavanols. Interestingly, a PEF pre-treatment on grapes was found to immediately reduce C6 compounds in juices at PM. After MAF, the young wine made from PEF-pre-treated-grapes contained more anthocyanins, stilbenes, and flavanols, more volatile esters, and a lower green odourant 1-hexanol than the untreated (no-PEF treated) counterpart. After MLF, wines produced from grapes treated with PEF at high specific energy retained higher levels of anthocyanins, stilbenes, flavonols, flavanols, hydroxycinnamic acids, and hydroxybenzoic acids. Overall, application of high specific energy and high electric field strength of PEF on Merlot grapes led to production of finished wines with a distinct phenolic composition and volatile profile.

As a function of storage time, an increment in the phenolic acids in conjunction with a decrement in the monomeric anthocyanins resulted in dulling the colour for all wines. Esters and acetates were the most reduced compounds in wines at 4°C over time. Degradation reactions including Maillard, anthocyanin cleavage, and hydrolysis were particularly accelerated at 45°C over time. Interestingly, only the wine made from grapes treated at high specific energy and high electric field strength had a decrease in anthocyanins when stored at 4°C. Storage at 25°C increased the phenolic acids in the wines made from PEF-treated grapes. At 45 °C, citronellol and 2-phenylethyl acetate in control wines were significantly reduced as a function of storage time. Meanwhile, only citronellol was lost at higher rates in wines made from grapes treated at high specific energy; and, only 2-phenylethyl acetate was diminished at faster rates in wines from grapes treated at low specific energy. More furan compounds were formed in control and wine obtained from grapes treated at low PEF intensity. Overall, PEF treatment applied to grapes at the initial stage of winemaking appeared to impact wine composition evident even after bottle storage.

These findings clearly evidenced the potential of pre-treating grapes with PEF to produce wines with unique characteristics at an industrial scale. Moreover, integrated targeted profiling and untargeted fingerprinting coupled with multivariate data analysis and discriminant marker selection was shown successfully, for the first time in the literature, as a useful tool to optimise electric field strength and specific energy of PEF treatments for winemaking application, as well as in describing the distinct properties of the wine produced.

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LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

*a**	green to red
AMDIS	automated mass spectral deconvolution and identification system
ANOVA	one-way analysis of variance
*b**	blue to yellow
C	saturation
C6 compounds	six-carbon compounds
CI	colour intensity
DVB/CAR/PDMS	divinylbenzene/carboxen/polydimethylsiloxane
h°	hue
HPLC-DAD	high performance liquid chromatography - diode-array detector
HS-SPME-GCMS	headspace – solid phase microextraction - gas chromatography/mass spectrometry
*L**	lightness
LAB	lactic acid bacteria
LV	latent variables
MAF	maceration-alcoholic fermentation
MLF	malolactic fermentation
MPP	Mass Profiler Professional
MVDA	multivariate statistical data analysis
NaOH	sodium hydroxide
NIST	National Institute of Standards and Technology
PCA	principal component analysis
PEF	pulsed electric fields
PLS-DA	partial least squares-discriminant analysis
PLS-R	partial least squares - regression
PM	pre-maceration
RI	retention index
TA	titratable acidity
TSS	total soluble solids
VID	variable identification coefficients

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CHAPTER 1

INTRODUCTION

1.1 Introduction

For over two decades of exporting, the New Zealand wine industry upholds world-leading position for producing high quality wines garnering $1.7 billion of total export value in 2018 (New Zeand Winegrowers Inc., 2018). Competitive growth of the wine industry demands continued development in winemaking technologies to produce high quality novel wines. The organoleptic properties of wines are influenced by the phenolic and volatile composition. Hence, an effective extraction of phenolic compounds and aroma precursors from the grape berry is crucial to the finished wine's colour, taste, mouthfeel and even health effects. Phenolic compounds in red wines include flavonoids such as anthocyanin, flavonol, flavanol, and flavanonol, and non-flavonoids including stilbene, hydroxycinnamic acid, and hydroxybenzoic acid (Ginjom, D'Arcy, Caffin, & Gidley, 2011). These compounds are particularly responsible for the astringency, bitterness, colour, and the antioxidant properties of wine (Casassa & Harbertson, 2014). Meanwhile, aroma precursors from the grapes transformed into volatile compounds through fermentation can be classified into esters, higher alcohols, terpenes, fatty acids, aldehydes, ketones, or norisoprenoids (Arcari, Caliari, Sganzerla, & Godoy, 2017). During winemaking, thousands of reactive phenolic and aroma precursors are greatly modified during alcoholic and malolactic fermentations through their interaction with other reactive species, enzymes, yeast, and lactic acid bacteria, and exposure to ethanol accumulation and changing pH and overall composition (Bartowsky, 2014; Bimpilas, Tsimogiannis, Balta-Brouma, Lymperopoulou, & Oreopoulou, 2015; Cheynier, Schneider, Salmon, & Fulcrand, 2010). Since (bio) chemical processes involved in the winemaking from grapes to wine are complex, a more comprehensive technique of analyses is needed to have a better understanding on the evolution of those compounds during the fermentation.

The application of pulsed electric fields (PEF) technology has recently been introduced to winemaking industries. This technology applies intermittent high voltage electric pulses for a short time (µs to ms) on a biomaterial to increase cell permeability by electropermeabilisation or electroporation, which facilitates mass transfer across cell membrane (Goldberg et al., 2018; Zimmermann, 1986). Researches on the use of PEF technology for winemaking are mostly conducted at laboratory scale. PEF treatment at low electric field strength (0.7 – 10 kV/cm) and specific energy (0.4 – 50 kJ/kg) applied to the grapes prior to maceration has demonstrated to increase and accelerate the release of phenolic compounds in grape juice (Delsart et al., 2014;

Donsì, Ferrari, Fruilo, & Pataro, 2010; El Darra, Rajha, et al., 2016; Leong, Oey, & Burritt, 2016; López-Alfaro et al., 2013; Teusdea, Bandici, Kordiaka, Bandici, & Vicas, 2017) and in fermenting juice after maceration – alcoholic fermentation (Donsì et al., 2010; El Darra, Rajha, et al., 2016; López-Giral et al., 2015; López, Puértolas, Condón, Álvarez, & Raso, 2008a, 2008b; López, Puértolas, Hernández-Orte, Álvarez, & Raso, 2009; Luengo, Franco, Ballesteros, Álvarez, & Raso, 2014; Puértolas, López, Saldaña, Álvarez, & Raso, 2010; Puértolas, Saldaña, Condón, Álvarez, & Raso, 2010; Saldaña et al., 2017). So far, there is limited study investigating the effect of PEF on the composition of finished wines after malolactic fermentation and on the volatile compounds of grape juice and wine, especially when the PEF treatment and winemaking conducted at a commercial scale. Therefore, there is a need to examine the global impact of PEF treatment of grapes prior to maceration on both phenolic and volatile composition, in addition to colour and oenological properties, at every winemaking stage to fully grasp the effects of PEF treatment.

In addition to fermentation, wine composition changes during storage. The storage stability of wine largely influenced by the initial composition of the wine, storage time, and storage temperature determines the extent of compositional changes which may be positive (as in maturation) or negative (as in deterioration) (Avizcuri, Sáenz-Navajas, Echávarri, Ferreira, & Fernández-Zurbano, 2016; Mattivi, Arapitsas, Perenzoni, & Guella, 2015; Scrimgeour, Nordestgaard, Lloyd, & Wilkes, 2015). Studies on post-bottling of wines made from PEF-treated grapes are limited and focused only on phenolic composition and colour during bottle ageing (Puértolas, Saldaña, Condón, Álvarez, & Raso, 2009; Puértolas, Saldaña, Condón, et al., 2010). Taking into account the potential effect of PEF pre-treatment on the composition of the finished wines, storage study on wines made from PEF-treated grapes is needed for establishing appropriate storage conditions to preserve their quality.

1.2 Objectives and approaches

This study primarily aims to evaluate the impact of PEF pre-treatment on the winemaking of Merlot wine. Four PEF conditions varying in electric field strength and specific energy were applied on grapes before maceration. Considering the complexity of the sample matrix (i.e. grape juice, fermenting juice, finished wine, and stored wine), targeted profiling of phenolic compounds, colour, and oenological attributes combined with untargeted fingerprinting of volatile composition of each sample were conducted. These comprehensive analyses of the sample attributes were coupled with multivariate data analyses and discriminant marker identification to determine important compounds significantly affected by PEF treatment or by the vinification steps and storage. To differentiate the influence of winemaking and storage and the effect of PEF pre-treatment, the following specific objectives were accomplished stepwise:

1. To evaluate the feasibility of using integrated fingerprinting, profiling and chemometrics approach to differentiate volatile composition, phenolic profile, colour, and oenological characteristics of grape samples at varying stages of winemaking (i.e. the grape juice at pre-maceration (PM), the fermenting juice after simultaneous maceration–alcoholic fermentation (MAF), and the wine after malolactic fermentation (MLF)). This study was discussed in *Chapter 3*. The technique is further used in *Chapters 4* and *5*.
2. To identify the major (bio) chemical changes during the winemaking stages: PM, MAF, and MLF. This study was discussed in *Chapter 3*.
3. To investigate the effect of different PEF pre-treatment conditions by comparing samples collected at PM, MAF, and MLF. This study was discussed in *Chapter 4* where integrated fingerprinting, profiling and chemometrics approach was utilised.
4. To investigate the effect of storage time, storage temperature, and PEF pre-treatment on the phenolic and volatile composition and oenological attributes of stored wines. This study was discussed in *Chapter 5* where integrated fingerprinting, profiling and chemometrics approach was utilised.

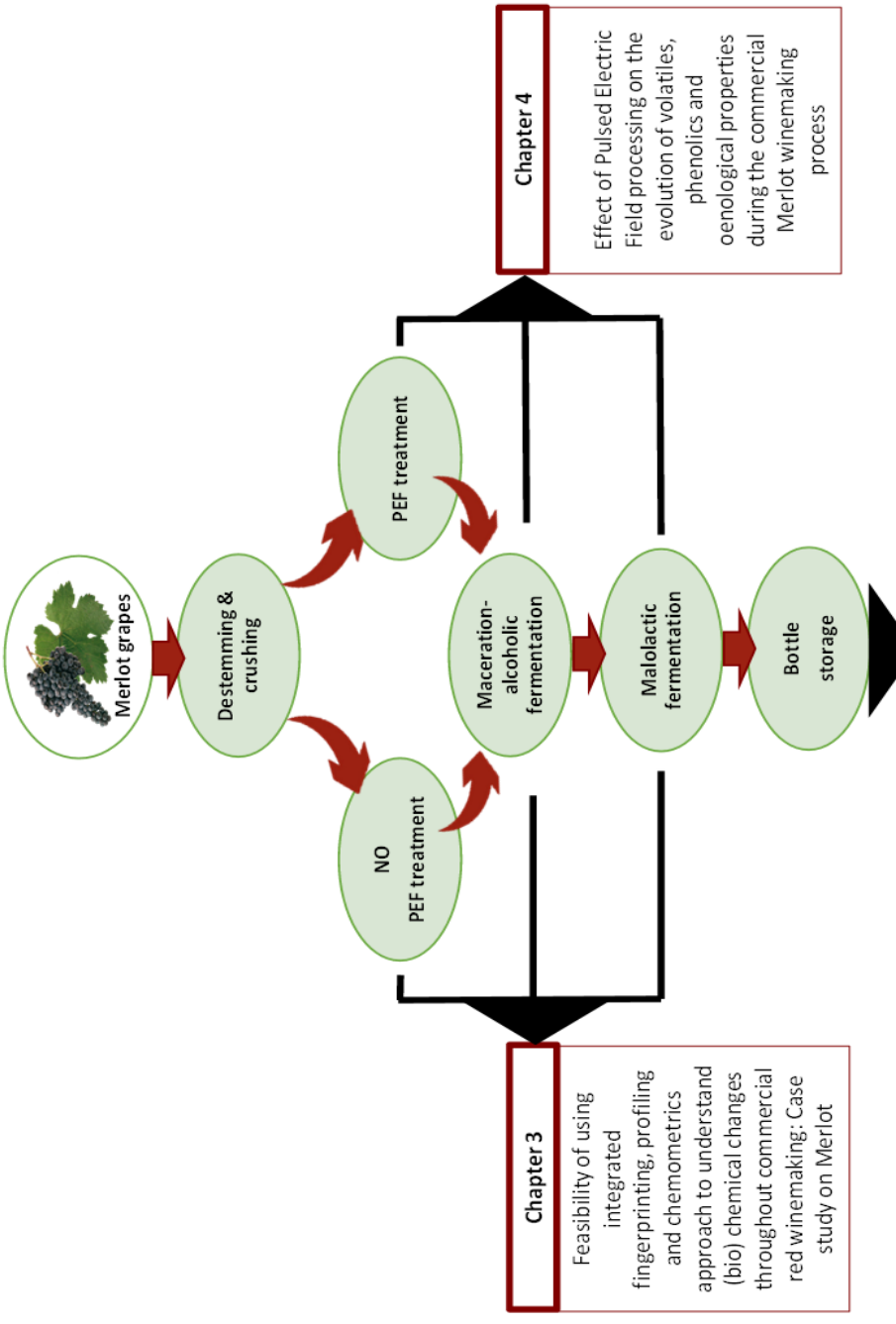

Fig. 1.1 Thesis sampling points and corresponding working chapter.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This literature review presents the characteristics of Merlot grapes and wine, and the effects of maceration techniques applied on grapes, in terms of polyphenols, aroma compounds/volatile profile, colour, physicochemical properties, and health related properties. Secondly, pulsed electric fields treatment is introduced along with the studies on the application of PEF on merlot grapes. Lastly, metabolic fingerprinting of wines from PEF treated Merlot grapes is discussed.

2.2 Merlot winemaking

Wines are evaluated by colour, aroma, and taste. These organoleptic properties of red wines are dictated by the plant's secondary metabolites extracted from the grape berry, such as phenolic compounds, aroma precursors, and volatile compounds, which evolves throughout winemaking. Phenolic compounds give wine its colour, taste, mouthfeel, and antioxidant properties (Teixeira, Eiras-Dias, Castellarin, & Gerós, 2013). Aroma precursors are bound, non-volatile compounds that develop into the volatile and odourous forms (Darriet, Thibon, & Dubourdieu, 2012).

In this section, the specific location of secondary metabolites in the merlot grape berry, their extraction through different maceration techniques used in winemaking and their evolution are discussed. Furthermore, merlot wine is described through its quality properties such as its volatile and phenolic profiles, colour, physico-chemical properties and its effect on health.

2.2.1 Plant metabolites and their location in *Vitis vinifera* cv. Merlot grape berry

Plant metabolites are not evenly distributed in the grape berry. The grape skins, pulp, and seeds contain different polyphenols, aroma precursors and volatile compounds at varying concentrations. Hence, the type and amount of plant metabolites available for winemaking depends on the degree of extraction happening on the different parts of the grape berry or whether any of the skin, pulp or seeds are excluded in the maceration process (Godevac et al., 2010). In *Vitis vinifera* grape berries, the seed contains most of the total soluble phenolics in the berry at about 60-70%, followed by the skin at 28-35%, and the pulp at $\leq$10% (Godevac et al., 2010). Volatile compounds and aroma precursors are mostly located in skins

and seeds of the grape berry (Darriet et al., 2012; González-Barreiro, Rial-Otero, Cancho-Grande, & Simal-Gándara, 2015).

Inside the plant cells of different parts of the grape berry, plant metabolites are stored in the cell vacuoles. Vacuoles play an important role in the exchanges of material and information with other plant cells and their environment. It stores sugars, organic acids, aromas, flavours, ions and water, which are all necessary for plant physiological processes such as homeostasis, protection against biotic and abiotic stresses, and pigmentation (Fontes, Gerós, & Delrot, 2011). Because of the varying functions of vacuoles, vacuoles in a plant system differ in morphology, biochemistry, and biogenesis. Consequently, vacuoles from different parts of the plant carry different compounds. Hence, degree of extraction of plant metabolites from different grape parts may vary.

Most of the phenolic compounds and aroma compounds are in their glycosylated forms (**Tables 2.1-2.3**). These forms allow the higher solubility, easier transport and increased storage ability inside the vacuoles (Fontes et al., 2011).

2.2.1.1 *Phenolic compounds*

The phenolic compounds in the grape berry can be grouped into flavonoids and non-flavonoids. Compounds belonging to the class of flavonoids are anthocyanins, flavonols, flavanols, proanthocynidins or tannins, flavanons and flavons. On the other hand, non-flavonoids are phenolic acids, particularly, hydroxybenzoic acids, hydroxycinnamic acids and other phenol derivatives such as stilbenes (Teixeira et al., 2013).

Anthocyanins are water-soluble pigments responsible for the red to blue colouration of the grape skins and, through vinification, in wines (Fernandes de Oliveira & Nieddu, 2016). Flavonols, mostly found in grape skins copigment with anthocyanins which stabilizes wine colour (Pantelić et al., 2016; Shi et al., 2016). Flavanols, found in seeds, also aid in stabilizing wine colour and contributes to the astringency and bitterness of wine. Polymers of flavanols, called proanthocyanidin or condensed tannins located in skins and seeds, are also responsible in the astringency and bitterness (Chira, Lorrain, Ky, & Teissedre, 2011). Ninety-five percent of the hydroxybenzoic acids, mostly gallic acid, is in the seeds (Pantelić et al., 2016). Small amounts of hydroxycinnamic acids are present in all grape berry.

Resveratrol, the most popular stilbene due to health effects, is located in skins (Pantelić et al., 2016).

Table 2.2.1 Polyphenols found in the skin of *Vitis vinifera* cv. Merlot grape berries

hydroxybenzoic acids	*anthocyanins*
gallic acid[a]	delphinidin-3-o-glucoside[b]
protocatechuic acid[a]	cyanidin-3-o-glucoside[b]
ellagic acid[a]	petunidin-3-o-glucoside[b]
dimer (epi)gallocatechin-(epi)catechin[b]	peonidin-3-o-glucoside[b]
hexose ester of protocatechuic acid[b]	malvidin-3-o-glucoside[b]
hexose ester of vanillic acid[b]	delphinidin-3-o-(6-o-acetyl)-glucoside[b]
syringetin-3-o-glucoside[b]	peonidin-3-o-glucoside-pyruvic acid[b]
	cyanidin-3-o-(6-o-acetyl)-glucoside[b]
hydroxycinnamic acids	petunidin-3-o-(6-o-acetyl)-glucoside[b]
chlorogenic acid[a]	delphinidin-3-o-(6-o-coumaroyl)-glucoside[b]
caffeic acid[a]	peonidin-3-o-(6-o-acetyl)-glucoside[b]
ferulic acid[a]	malvidin-3-o-(6-o-acetyl)-glucoside[b]
trans-cinnamic acid[b]	peonidin-3-o-(6-o-caffeoyl)-glucoside[b]
	cyanidin-3-o-(6-o-coumaroyl)-glucoside[b]
coumarins	petunidin-3-o-(6-o-coumaroyl)-glucoside[b]
aesculin[a]	peonidin-3-o-(cis-6-o-coumaroyl)- glucoside[b]
hexose ester of ferulic acid[b]	malvidin-3-o-(cis-6-o-coumaroyl)-glucoside[b]
	peonidin-3-o-(trans-6-o-coumaroyl)-glucoside[b]
flavonols	malvidin-3-o-(trans-6-o-coumaroyl)-glucoside[b]
kaempferol[a]	
quercetin[a]	*flavanons*
rutin[a]	naringin[a]
myricetin[a]	
quercetin-3-o-hexoside[b]	*flavons*
myricetin-3-o-glucuronide[b]	apigenin[a]
isorhamnetin-3-o-galactoside[b]	
myricetin-3-o-glucoside[b]	*stilbenes*
dihydroquercetin-3′-o-rhamnoside[b]	resveratrol[a, b]
quercetin-3-o-galactoside[b]	
quercetin-3-o-glucuronide[b]	*flavan-3-ols*
quercetin-3-o-glucoside[b]	gallocatechin[a]
laricitrin-3-o-glucoside[b]	epigallocatechin[a]
kaempferol-3-o-galactoside[b]	catechin[a]
kaempferol-3-o-glucoside[b]	gallocatechin gallate[a]
isorhamnetin-3-o-glucoside[b]	catechin 3-gallate[a]
quercetin-3-o-rutinoside[b]	gallocatechin[b]
	procyanidin dimer[b]
	(+)-catechin[b]

[a]Pantelić et al. (2016)
[b]Shi et al. (2016)
[c]Chira et al. (2011)

Table 2.2.2 Polyphenols located in the pulp and seed of *Vitis vinifera* cv. Merlot grape berry

Pulp	**Seeds**	
hydroxybenzoic acids	*hydroxybenzoic acids*	*flavonols*
gallic acid[a]	gallic acid[a]	kaempferol[a]
protocatechuic acid[a]	protocatechuic acid[a]	quercetin[a]
p-hydroxybenzoic acid[a]	p-hydroxybenzoic acid[a]	rutin[a]
gentisic acid[a]	gentisic acid[a]	myricetin[a]
	ellagic acid[a]	
hydroxycinnamic acids		*flavanons*
chlorogenic acid[a]	*hydroxycinnamic acids*	naringin[a]
caffeic acid[a]	chlorogenic acid[a]	
ferulic acid[a]	caffeic acid[a]	*flavan-3-ols*
coumarins	ferulic acid[a]	epigallocatechin[a]
aesculin[a]	*Coumarins*	Catechin[a]
	aesculin[a]	Epicatechin[a]
flavan-3-ols		gallocatechin gallate[a]
epigallocatechin[a]		catechin 3-gallate[a]
gallocatechin gallate[a]		epigallocatechin gallate[a]
epigallocatechin gallate[a]		
flavonols		
rutin[a]		
flavanons		
hesperetin[a]		

[a]Pantelić et al. (2016)
[b]Shi et al. (2016)
[c]Chira et al. (2011)

2.2.1.2 ***Aroma precursors and volatile compounds***

Merlot is among the non-aromatic red wine grape varieties having only trace amounts of aromatic compounds (ng/L - mg/L) (Song et al., 2016). Some of the important, most odourous ones are present at very low concentrations (Darriet et al., 2012).

Grape berries contain free or volatile aroma compounds and the bound aroma precursors normally found as glycosides, which upon release through vinification, evolve into odour-active compounds (Pedroza, Zalacain, Lara, & Salinas, 2010). Most of the glycosides are located in the skin. These are composed of aglycones of alcohols, esters, acids, terpenes, ketones, and aldehydes, which are linked with disaccharides, namely, glucose-rhamnose, arabinose and apiose (Pedroza et al., 2010).

Table 2.2.3 Free and bound aroma compounds in Merlot grape berry

Free volatile compounds	Glycosidically bound volatile compounds
C6 compounds	*C6 compounds*
hexanal[a]	1-hexanol[a]
trans-2-hexenal[a, b]	
(E)-2-hexenal[b]	*alcohols*
1-hexanol[a]	1-heptanol[a]
	1-octen-3-ol[a]
aldehydes and alcohols	1-octanol[a]
heptanal[a]	1-decanol[a]
trans-2-octenal[a]	1-hexanol[a]
decanal[a]	
1-octanol[a]	*terpenes*
octanal[a]	cis-furan-linalool oxide[a]
nonanal[a]	nerol[a]
1-octen-3-ol[a]	trans-furan-linalool oxide[a]
	geraniol[a]
terpenes	linalool[a]
limonene[a, b]	α-terpineol[a]
linalool [a, b]	4-terpineol[a]
α-terpineol [a, b]	
geranyl acetone[a, b]	*C13-norisoprenoids*
farnesol[b]	vitispirane[a]
	β-damascenone[a]
	1,1,6-trimethyl-1,2-dihydronaphthalene
C13-norisoprenoids	(TDN) [a]
vitispirane[a]	3-hydroxy-β-damascenone[a]
β-damascenone[a, b]	3-hydroxy-7,8-dihydro-β-ionol[a]
β-ionone[a, b]	3-oxo-α-ionol[a]
dihydroactinidiolide[a]	
3-hydroxy-β-damascenon[a]	*Shikimic acid derivatives*
	2-methoxy-4-methylphenol[a]
alkylated methoxypyrazine	benzyl alcohol[a]
3-isobutyl-2-methoxypyrazine (IBMP) [d]	2-methoxy-4-vinylphenol[a]
	benzaldehyde[a]
	2-phenylethanol[a]

[a] Song, Shellie, Wang, & Qian (2012)
[b] Pedroza, Zalacain, Lara, & Salinas (2010)
[c] Darriet, Thibon, & Dubourdieu (2012)
[d] Sivilotti et al. (2016)

Aroma compounds can, then, be classified into the volatile and odourous compounds (higher concentrations than odour thresholds) and the odourless, non-volatile aroma precursors (Darriet et al., 2012). Volatile and odourous aroma compounds include alkylated methoxypyrazines, terpense, C-13 noriseprenoids, and volatile thiols. The non-odourous, non-volatile and bound aroma precursors are unsaturated fatty acids, phenolic acids, and the

glycosides (Darriet et al., 2012). However, only a few of these compounds have been studied and detected in Merlot grape berries (**Table 2.3**).

2.2.2 Winemaking process

Winemaking starts in the preparation of grapes by destemming and crushing. Maceration – alcoholic fermentation follows where the grape skins, seeds and, sometimes, stems are soaked together for a period of time to extract plant metabolites into the fermenting must. After the controlled maceration period, the skins and seeds are separated from the partly fermented grape by pressing. The must will undergo alcoholic fermentation where sugars are converted to ethanol and carbon dioxide. Malolactic fermentation may be performed to reduce the concentration of harsh malic acid by lactic acid bacteria conversion to softer lactic acid and carbon dioxide. Lastly, the wine is aged for maturation and bottling.

Simultaneously, phenolic compounds, aroma precursors, and volatile compounds change upon interaction with each other.

2.2.2.1 *Maceration techniques used on Merlot*

All phenolic and aromatic compounds necessary to create the desired colour, aroma and taste of the wine are extracted from the grapes through the maceration process. Hence, effective and selective extraction of phenolic and aromatic compounds is critical to the quality of wine.

As shown in **Table 2.4**, several maceration techniques and treatments prior to maceration has been applied on Merlot grapes such as utilizing enzymes, cold maceration, microwave treatment, dehydration, and pulsed electric fields treatment. These studies demonstrate the effects of different techniques, although analyses of these effects were not followed through each step of winemaking. Most of the significant effects (including increased extraction or concentration of phenolic compounds) due to techniques such as dehydration and PEF were apparent during maceration stage.

Table 2.4. Effect of different maceration techniques on the chemical composition of Merlot must or wine at different stages of winemaking

Maceration technique	Parameters	Effect of different maceration techniques with respect to traditionally macerated samples				References
		Maceration	Alcoholic fermentation	Malolactic fermentation	Ageing	
maceration enzymes	commercial enzyme Rapidase® Ex Colour (DSM, Delft, the Netherlands) at 2.5 g/100 kg grape berries		(-)16% anthocyanins, ns			(González-Neves, Favre, Piccardo, & Gil, 2016)
pectolytic enzymes	Vinozym FCE-G (Novo Nordisk, Dittingen, Switzerland) at 2.5 g/100 kg grape berries			(=) anthocyanins		(Poussier, Guilloux-Benatier, Torres, Heras, & Adrian, 2003)
cold maceration	5 days at 10–15 °C		(=)6.22% anthocyanins			(González-Neves, Favre, Piccardo, & Gil, 2016)
cold maceration	4 days at 8°C		(+)20% esters; (+)43% octanoic acid; (+)67% decanoic acid; (+)>20% α-terpineol and geraniol; (+)16% polyphenols, (+)8% anthocyanins			(De Santis & Frangipane, 2010)
cold maceration	60 hr at 7°C			(=) anthocyanins; (=) tannins		(Poussier, Guilloux-Benatier, Torres, Heras, & Adrian, 2003)
microwave	power = 200W, t = 420s, T = 47.4–49°C.; 3 days at room temperature		(+)64.61% phenols			(Bandici, Vicaş, Teuşdea, Bandici, & Popa, 2017)

Abbreviations: t, treatment time; T, application temperature (°C). (+) increase, (-) decrease or (=) no significant difference in concentration in comparison with the traditionally macerated samples.

Table 2.4. *Continuation*

Maceration technique	Parameters	Effect of different maceration techniques with respect to traditionally macerated samples				References
		Maceration		Alcoholic fermentation	Malolactic fermentation	Ageing	
dehydration	35% relative humidity at 7°C; 30% and 40% weight loss.	30%	hydrobenzoic acid: (-)gallic, (-)vanillic, (-)ellagic; hydroxycinnamic acid: (-)caffeic, (-)p-coumaric, (-)ferulic; flavanols: (+)catechin, (-)epicatechin); flavonols: (-)myricetin, (-)quercetin, (-)kaempferol); (+)trans-resveratrol				(Panceri, Gomes, De Gois, Borges, & Bordignon-Luiz, 2013)
		40%	hydrobenzoic acid: (-)gallic, (-)protocatechuic, (-)vanillic, (-)syringic, (-)ellagic; hydroxycinnamic acid: (+)caffeic,(-)p-coumaric, (-)ferulic; flavanols: (+)catechin, (+)epicatechin; flavonols: (+)myricetin, (-)quercetin, (-)kaempferol; (+)trans-resveratrol; (+)tyrosol				(Panceri, Gomes, De Gois, Borges, & Bordignon-Luiz, 2013)

Abbreviations: (+) increase, (-) decrease or (=) no significant difference in concentration in comparison with the traditionally macerated samples.

Table 2.4. *Continuation…*

Maceration technique	Parameters	Effect of different maceration techniques with respect to traditionally macerated samples				References
		Maceration	Alcoholic fermentation	Malolactic fermentation	Ageing	
PEF	d = 0, 2, 4, 8 and 14 days, E = 1.5, $N_{PEF\ low}$ = 243, $N_{PEF\ high}$ = 1033, t = n.d., P = square, f = 50, τ = 20, $W_{PEF\ low}$ = 14.68, $W_{PEF\ high}$= 69.76, batch	Anthocyanins - PEF low: n.d., (+)280%, (+)157%, (+)90%, (+)26%; PEF high: n.d., (+)373%, (+)190%, (+)100%, (+)15% Total phenolics – PEF low: (+)7%, (+)80%, (+)61%, (+)63%, (+)20%; PEF high: (+)69%, (+)106%, (+)71%, (+)36%, (+)17%				(Leong, Burritt, & Oey, 2016)
PEF	E = 7, N = 50, t = 0.15ms, P = square, f = 122, τ = 3, W = 3.67, continuous		(+)7% anthocyanins			(Puértolas, López, Saldaña, Álvarez, & Raso, 2010)
PEF	E = 0.5, N = 1000, t = 100ms, P = square, f = n.d, τ = 100, W = n.d., batch			(+)25% anthocyanins		(Delsart et al., 2012)

Abbreviations: t, treatment time; T, application temperature (°C); E, electric field strength (kV/cm);N, pulse number; P, pulse shape (exponential decay or square waveform); f, pulse frequency (Hz); τ, pulse width (μs); W, specific energy input (kJ/kg); batch or continuous mode of treatment. n.d., no data available. (+) increase, (-) decrease or (=) no significant difference in concentration in comparison with the traditionally macerated samples.

2.2.2.2 *Evolution of volatile compounds and aroma*

Wine aroma develops using the aroma compounds (free and bound) in grape berries as starting material. Chemical reactions and biochemical processes happening after their release from grape cells through maceration, during alcoholic and malolactic fermentation, and aging change and add complexity into the aroma of wine. Aroma compounds present in wines can be classified according to their origin, namely grape or varietal aroma, fermentation aroma, and aging aroma (Ilc, Werck-Reichhart, and Navrot, 2016).

Chemical and biochemical processes that modify the profile of aroma compounds include deglycosylation, enzymatic action, yeast consumption, spontaneous transformation, photooxygenation, and thermal degradation. **Figure 2.1** shows the significant difference between the amount of free and bound forms of selected classes of aroma compounds (e.g. acids, monoterpenes, norisoprenoids, volatile phenols, and benzenoids) between grapes and wines (of different varieties), due to deglycosylation alone (Ilc et al., 2016). Moreover, wines showed an increased total amount of aroma compounds where, within each class, individual compounds vary in the extent of deglycosylation. For instance, glycosylated forms of linalool oxides detected in grapes and must turn into odour-active forms in wines after deglycosylation during winemaking (Ilc et al., 2016).

Consequently, the presence and amount of individual compounds do not follow a consistent increase or decrease along vinification. These inconsistencies were observed as significant variations of the aroma descriptions of Merlot wine every day of maceration (Pineau, Barbe, Van Leeuwen, & Dubourdieu, 2011), difference in amount of free and bound forms of aroma compounds after fermentation (Ilc et al., 2016), and fluctuations of the amount of aroma compounds every week of accelerated wine ageing (Loscos, Hernández-Orte, Cacho, & Ferreira, 2010).

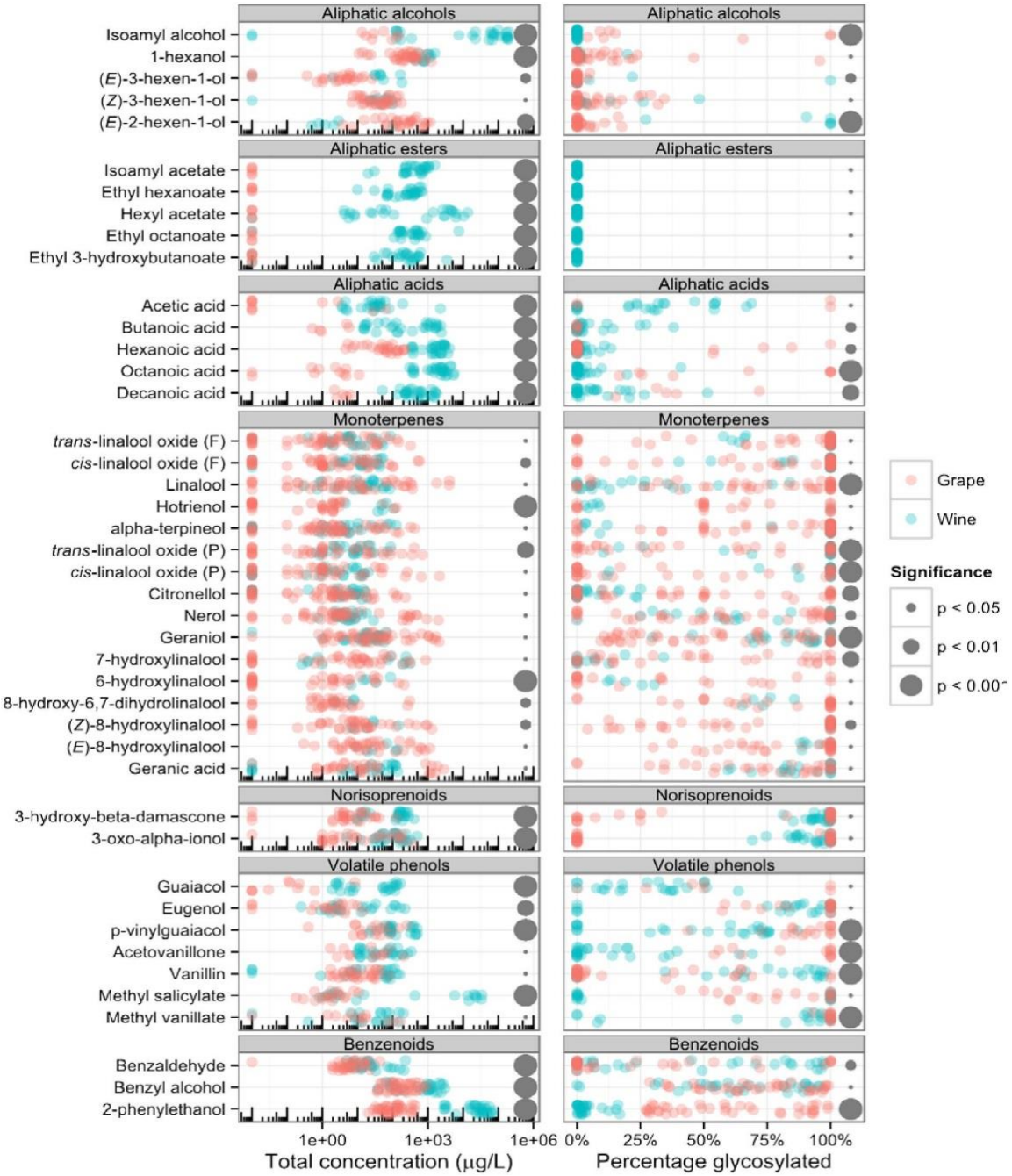

Figure 2.1 Difference between the total concentration (left) and percentage of glycosylation (right) of aroma compounds in grape and wines. Total concentration is the sum of free and bound aroma compound concentration. Percentage glycosylated is bound concentration divided by total concentration. Each points are values from grape and wine samples used in 19 publications analysed by Ilc, Werck-Reichhart, and Navrot (2016). Gray dots on the right of both columns of graph indicate significant difference between grapes and wine, with their size proportional to the p-value of the statistical test used. Lifted from Ilc et al. (2016).

2.2.2.3 *Evolution of polyphenols*

Winemaking processes influences the phenolic compounds composition of the wines. Overall, wines contain less amount of phenolic compounds compared to the grape must after optimum maceration.

Studies in merlot grape and wine have shown that anthocyanins, a flavonoid, change concentration due to transformation. These transformations include oxidation reactions González-Neves, Gil, and Barreiro (2008), formation of anthocyanin derived pigments that can be due to reactions with yeast metabolites, and adsorption to yeast cell walls (Lingua, Fabani, Wunderlin, & Baroni, 2016). Malvidin and its derivatives are also observed remain as the most abundant anthocyanin in grape berries and wine due to its stability (Lingua et al., 2016).

After winemaking, glycosylated flavonols decrease because of acid hydrolysis into their corresponding free aglycons. Moreover, glycosylated forms are also lost due to oxidation, copigmentation with anthocyanins, as well, as adsorption to yeast cell walls. Hydrolysis of the main flavonol glycosides from grapes produces quercetin, making it the most abundant flavonol (Lingua et al., 2016).

In the study of Lingua et al. (2016), flavanols slightly increased after winemaking was attributed to the further hydrolysis of its precursors.

Nonflavonoid compounds also have varying trends. Hydroxycinnamic acid decreased despite hydrolysis of its bound form, while hydroxybenzoic acid increased. The important stilbene resveratrol concentration at the end of winemaking is influenced by the losses through absorption into the yeast cells and addition through further hydrolysis of its glycosidic forms (Lingua et al., 2016).

Along with phenolic composition and concentration, antioxidant activity and colour also changes depending on the change in concentration and the dominant anti-oxidative compounds (Lingua et al., 2016).

2.2.3 Quality properties of Merlot wine

Parameters in evaluating and characterizing wines include its volatile profile, polyphenols, colour index, and physico-chemical properties such as pH, total acidity, total soluble solids, and alcohol concentration.

2.2.3.1 *Volatile profile of Merlot wine*

The aroma of wine is composed of more than a hundred odourless and odour-active volatile compounds. Volatile compounds without odour or at concentrations below odour thresholds can still indirectly affect the aroma of wine (Darriet et al., 2012). Studies have shown that the volatile profile of Merlot wine is composed of acids, fatty acids, esters, higher alcohols, terpenes, sulphur compounds, lactones, aldehydes, vanillin derivatives, C-13 norisoprenoids, phenols, and other classes (**Table 2.5**). Among the acids identified in the volatile fraction of merlot wines are acetic acid, malic acid, and succinic acid (Varela, Barker, Tran, Borneman, & Curtin, 2017).

Table 2.5 lists the volatile compounds and their effect on the aroma of the merlot wine at concentrations higher than their odour thresholds.

Table 2.2.5 Volatile compounds in Merlot wine and their contribution to the wine's aroma

Compounds	Aroma descriptor	Odour threshold (µg/L)
Volatile fatty acids		
isobutyric acid[a, c, f]	cheese, rancid, fat	8 100 - 200 000
butyric acid[b]	cheese, rancid	10 000
hexanoic acid[a, c, d, f]	cheese, greasy	420 – 3 000
octanoic acid[a, c, d, f]	rancidity, candy, cheese, animal, spicy, unpleasant	500
propanoic acid[a, d]	cheese, vinegarish	8 100
3-methyl-butanoic acid[d]	unpleasant	unknown
nonanoic acid[d]	coconut, fatty	unknown
n-decanoic acid[a, d, f]	sour, fatty, unpleasant	1 000
9-decenoic acid[d]	fruity frankincense	1 000
dodecanoic acid[d]	laurel oil	unknown
thioacetic acid[a]	toasted	unknown
isovaleric acid[a]	candy, cheese, rancidity	3 000
2-methyl butyric acid[f]		50
Volatile esters		
ethyl acetate[a, d, e, f]	solvent, fruity, balsamic	7 500 - 12 000
ethyl propanoate[a, e]	fruity, solvent, acetone	10
2-methylpropyl acetate[d, e]	fruity, green	
ethyl butanoate[a, e]	fruity, strawberry	400
ethyl 2-methyl butanoate[a, e]	strawberry, candy fruit	18
ethyl 3-methyl butanoate/ butanoic acid 3-methyl-ethyl ester[c, e]	fruity, floral	unknown
2-methylbutyl acetate[e]		unknown
ethyl hexanoate[a, c, d, e, f]	fruity, green apple, strawberry, spicy and anise	14
hexyl acetate[a, e]	fruity, herbs, apple, pear, cherry	670
ethyl octanoate[a, c, d, e, f]	fruity, candy, pineapple, pear, floral	5 - 580
ethyl decanoate[a, c, d, e, f]	fruity, fatty, pleasant	200
isoamyl lactate/ 1-butanol, 3-methyl-, acetate[c, d]	banana	30
ethyl lactate[a, c, f]	acid, medicinal, strawberry, raspberry	14 000 – 250 000
ethyl 4-hydroxybutyrate[c]	caramel, cotton candy	unknown
diethyl succinate[a, c, d, f]	overripe melon, lavender	100 000 – 200 000
diethyl malate[c]	green	760 000

[a]Arcari et al. (2017)
[b]Hernandez-Orte et al. (2014)
[c]Panceri, Burin, Caliari, Amboni, and Bordignon-Luiz (2017)
[d]Song et al. (2016)
[e]Varela et al. (2017)
[f] Zhang, Tao, Wen, and Wang (2013)

Table 2.5. *Continuation…*

Compounds	Aroma descriptor	Odour threshold (µg/L)
Volatile esters		
2-phenylethyl acetate[d]	pleasant, floral	650
3-methyl ethyl propionate[d]	sweet, fruity	unknown
ethyl butyrate[b, d, f]	pineapple, banana	20
ethyl-9-decenoate[a, d]	rose	100
ethyl phenylacetate[d, f]	honey	250 - 600
ethyl phenylpropiolate[d]	sweet fruit, honey, floral	unknown
benzoic acid, 2,5-dihydroxy-, methyl ester/ methyl 2,5-dihydroxybenzoate[d]	phenol, slightly bitter	unknown
N-acetyl-L-phenylalanine ethyl ester[d]	vanilla, cinnamon, fruits	unknown
ethyl succinate[d]	pleasant	unknown
ethyl citrate[d]	berry	unknown
ethyl laurate[d, f]	flowery, fruity	1 500
ethyl cinnamate[a, b, d]	honey, floral	unknown
ethyl hexadecanoate[d]	cream	1 500
ethyl myristate[a, d, f]	coconut, sweet	2 000
p-hydroxycinnamic acid ethyl ester[d]	spicy, cherry, apricot, honey	unknown
á-phenylethyl butyrate[d]	green, fruit, fragrance, roses	unknown
pentanoic acid, 2-phenylethyl ester[d]	apple	unknown
isobutyl acetate[a, b]	fruity, apple, banana	16 000
butyl acetate[b]		unknown
ethyl-2-methylbutyrate[b]		unknown
ethyl isovalerate[a, b, f]	fruity, apple	1 - 3
linalool acetate[b]		unknown
ethyl furoate[b]		unknown
ethyl dihydrocinnamate[b]		unknown
ethyl isobutanoate[a]	fruity, banana	15
isoamyl acetate[a]	banana	30
ethyl pentanoate[a]	fruity, apple	5
isopentyl isobutanoate[a]	candy, pineapple, banana	unknown
methyl octanoate[a]	fruity, citric	200

[a]Arcari et al. (2017)
[b]Hernandez-Orte et al. (2014)
[c]Panceri et al. (2017)
[d]Song et al. (2016)
[e]Varela et al. (2017)
[f] Zhang et al. (2013)

Table 2.5. *Continuation…*

Compounds	Aroma descriptor	Odour threshold (µg/L)
Volatile esters		
furfuryl acetate[a]	toasted	540
ethyl nonanoate[a]	fruity, floral	1 300
ethyl-3-hydroxybutanoate[a]	green grape, marshmallow	20 000
methyl salicylate[a]	peppermint	0.1
phenylethyl acetate[a, f]	roses, floral, honey	250
diethyl glutarate[a]	cotton candy	unknown
ethyl dodecanoate[a]	candy, floral, waxy, soap	1 500
ethyl palmitate[a, f]	waxy, greasy	1 500
isopentyl acetate[f]		30
2-o-2-phenylethyl formate[f]		unknown
ethyl 2-hydroxy-3-methyl butyrate[f]		1 000
isopentyl lactate[f]		200
isopentyl octanoate[f]		125
ethyl decenoate[f]		100
butyl butyrate[f]		100
Higher alcohols		
ethanol[e, f]		unknown
glycerol[d]		unknown
butanol[a, e]	medicinal	150 000
2-methyl propanol[e]		unknown
2,3-methyl butanol[e]		unknown
hexanol[a, c, d, e, f]	grass just cut	110 – 8 000
isoamyl alcohol[c]	wine, ripe fruit	30 000
trans-3-hexenol[c]	green	1000
trans-2-hexenol[c]	green tomato	400
cis-3-hexenol[c]	green, kiwi	400
benzyl alcohol[a, c, d, f]	floral, rose, phenolic, balsamic, citrusy, sweet	200 000
2-phenylethyl alcohol[a, c]	rose, talc, honey	10 000 – 14 000
3-phenyl-1-propanol (benzenepropanol)[a, c]	fruity, strawberry	unknown
3-methylthiopropanol[c]	cooked vegetables	500
1-propanol[a, d, f]	vinegarish	50 000 – 306 000
isobutanol[d, f]	fusel, alcohol	40 000

[a]Arcari et al. (2017)
[b]Hernandez-Orte et al. (2014)
[c]Panceri et al. (2017)
[d]Song et al. (2016)
[e]Varela et al. (2017)
[f] Zhang et al. (2013)

Table 2.5. *Continuation…*

Compounds	Aroma descriptor	Odour threshold (µg/L)
Higher alcohols		
2-phenylethyl alcohol[a, c]	rose, talc, honey	10 000 – 14 000
3-phenyl-1-propanol (benzenepropanol)[a, c]	fruity, strawberry	unknown
3-methylthiopropanol[c]	cooked vegetables	500
1-propanol[a, d, f]	vinegarish	50 000 – 306 000
isobutanol[d, f]	fusel, alcohol	40 000
1-pentanol[d]	fruity, balsamic	80 000
3-methyl-1-butanol[a, d]	applejack, spicy	30 000
3-methyl-1-pentanol[d, f]	fruity, floral	500
1-nonanol	fatty-floral	58
1-heptanol[d, f]	fruity, mouldy, musty	250
1-octanol[d, f]	intense citrus, roses	900
phenylethyl alcohol[d]	sweet rose	14 000.0
lauryl alcohol[d, f]	nut odour, metal odour	1 000.0
2,2-dimethyl-1-propanol[a]	alcohol, candy	25 000.00
3-hexen-1-ol[a]	green, fat	1 000
1-undecanol[a]	tangerine	unknown
furfuryl alcohol[a]	burnt	2 000
3-methyl-thio-1-propanol[a]	boiled vegetables	500
1-hexadecanol[a]	floral, waxy	unknown
isopentanol [f]		30 000
isohexyl alcohol [f]		5 000
2-heptanol [f]		250
(*E*)-3-hexen-1-ol [f]		400
(*Z*)-3-hexen-1-ol [f]		400
(*E*)-2-hexen-1-ol [f]		400
3-ethoxy-1-propanol [f]		100
2-ethyl hexanol [f]		8 000
2,3-butanediol [f]		120 000
3-methyl-1-propanol [f]		1 000
1-decanol [f]		400
β-phenyl-ethanol [f]		14 000
Terpenes		
linalool oxide a[c]	flower, creamy, earthy	65
linalool oxide b[c]	flower, creamy, earthy	7

[a]Arcari et al. (2017)
[b]Hernandez-Orte et al. (2014)
[c]Panceri et al. (2017)
[d]Song et al. (2016)
[e]Varela et al. (2017)
[f] Zhang et al. (2013)

Table 2.5. *Continuation…*

Compounds	Aroma descriptor	Odour threshold (µg/L)
Terpenes		
linalool[b, c, f]	flowery, muscat	25.2
linalool oxide C[c]	flower, creamy, earthy	unknown
linalool oxide D[c]	flower, creamy, earthy	unknown
geraniol[a, b, c]	geranium, rose	30
nerol[c]	sweet, flowery	400
α-terpineol[a, b, c]	pine, lily, anise, mint	250
exo-2-hydroxycineole[c]	eucalyptol	unknown
trans-geranic acid[c]	fruity	unknown
trans-8-hydroxylinalool[c]		unknown
cis-8-hydroxylinalool[c]		unknown
7-hydroxy-geraniol[c]		unknown
ho-diendiol (I) [c]		unknown
thymol[d]	spicy, kusaka	unknown
farnesol[d]	*Convallaria majalis*	unknown
citronellol[d]	green lemon	100
cedrol[d]	cedar wood	unknown
β-citronellol[b, f]		unknown
limonene[a]	lemon, orange	15
(E) β-ocimene[a]	candy, herbaceous	1800
linalool oxide[a, f]	floral	25
β-terpineol[f]		250
trans-geraniol[f]		36
[*E*]-nerolidol[f]		700
Sulphur compounds		
carbon disulfide[e]		unknown
dimethyl sulfide[e]		unknown
ethanethiol[e]		unknown
hydrogen sulfide[e]		unknown
methanethiol[e]		unknown
methyl thioacetate[e]		unknown
Lactones		
γ-butyrolactone[c]	coconut, caramel	35
4-carboethoxy-gamma-butyrolactone[c]	sweet, coconut	400
trans-whisky lactone[c]		unknown
γ-nonalactone[a, b]	coconut, peach	30

[a]Arcari et al. (2017)
[b]Hernandez-Orte et al. (2014)
[c]Panceri et al. (2017)
[d]Song et al. (2016)
[e]Varela et al. (2017)
[f]Zhang et al. (2013)

Table 2.5. *Continuation…*

Compounds	Aroma descriptor	Odour threshold (µg/L)
Lactones		
δ-decalactone [b]		unknown
γ-decalactone [b]		unknown
aldehydes		
2-furfural[c]	toasted almond	14 100
benzaldehyde[a, c, d]	bitter almond, nutty	200
phenylacetaldehyde [b]		unknown
syringaldehyde [b]		unknown
furfural[a]	bread, almonds, candy	14 000
Vanillin derivatives		
vanillin [b, c]	vanilla	60
ethyl vanillate[a, b, c]	pollen, flowery, vanilla	990
vanillic acid[c]	vanilla	unknown
acetovanillone [b, c]	sweet spices, honey-like	1 000
zingerone (4-(4-hydroxy-3-methoxyphenyl)-2-butanone) [c]	sweet, fruity, ginger	
methyl vanillate [b]		unknown
syringaldehyde [b]		unknown
Norisoprenoids		
actinidols 1 isomer[c]	camphor	unknown
actinidols 2 isomer[c]	camphor	unknown
3-hydroxy-β-damascone[c]	tea, tobacco	unknown
3-oxo-α-ionol[c]	apricot, honey, tobacco	unknown
3-hydroxy-7.8-dihydro-β-ionol[c]		unknown
vomifoliol[c]	fruity	unknown
β-damascenone[a, b, d, f]	honey, sweet	0.05
β-ionone[a, b, f]	violet, balsamic, roses	0.09
α- ionone[a, f]	fruity, floral, raspberry, violet	2.6
Phenols		
acetoin[c]	sour yogurt, sour milk	150 000
phenol, 2,4-bis(1,1-dimethylethyl)- [d]	phenolic	2 000
guaiacol [b]		unknown
o-cresol [b]		unknown
4-ethylguaiacol [b]		unknown

[a]Arcari et al. (2017)
[b]Hernandez-Orte et al. (2014)
[c]Panceri et al. (2017)
[d]Song et al. (2016)
[e]Varela et al. (2017)
[f]Zhang et al. (2013)

Table 2.5. *Continuation…*

Compounds	Aroma descriptor	Odour threshold (µg/L)
Phenols		
m-cresol [b]		unknown
eugenol [b]		unknown
4-ethylphenol [b]		unknown
4-vinylguaiacol [b]		unknown
2,6-dimethoxyphenol [b]		unknown
4-vinylphenol [b]		unknown
4-allyl-2,6-dimethoxyphenol [b]		unknown
2,4-di-tert-butyl-phenol [f]		200
Others		
naphthalene [d]	camphor	unknown
1,1,6-trimethyl-1,2-dihydronaphthalene[a]		unknown

[a]Arcari et al. (2017)
[b]Hernandez-Orte et al. (2014)
[c]Panceri et al. (2017)
[d]Song et al. (2016)
[e]Varela et al. (2017)
[f]Zhang et al. (2013)

2.2.3.2 *Polyphenols compounds of Merlot wine*

Aside from colour, astringency and bitterness, polyphenols also influence the aroma composition of wine as an aroma precursor and through their interaction with the aroma compounds (Cheynier et al., 2010). Hence, polyphenol profile of merlot wine is not only important for its characterization, but also in understanding the volatile profile and aroma of the wine. Identified polyphenols positively found in merlot wines are tabulated (**Table 2.6**).

Table 2.2.6 Polyphenols in Merlot wine

Anthocyanins	*Hydroxycinnamic acids*
acetylvitisin B[c]	caftaric acid [c]
carboxypyranomalvidin-3-gluc[c]	t-fertaric acid [a, b, c]
delphinidin-3-(6″-cou)glu[c]	c-coutaric acid [a, c]
delphinidin-3-glu[a, c]	t-coutaric acid [a, c]
malvidin-3-(6″-p-cou)glu[c]	p-coumaric acid derivative 1 [c]
malvidin-3-glu[a, c]	p-coumaric acid derivative 2 [c]
malvidin-3-glucose-ethyl(epi)catechin1[c]	p-coumaric acid derivative 3 [c]
malvidin-3-glucose-ethyl(epi)catechin2[c]	trans-caftaric acid [a]
peonidin-3-(6″-cou)gluc[c]	caffeic acid [a]
peonidin-3-acetylgluc[a, c]	p-coumaric acid [a]
petunidin-3-acetylgluc[a, c]	ethyl caffeate [a]
petunidin-3-glu[a, c]	ethyl coumarate [a]
pyrano-malvidin-3-gluc[c]	cinnamic acid [b]
pyrano-malvidin-3-p-cougluc[c]	Hx-es-fa, hexose ester of ferulic acid [b]
peonidin-3-glu[a]	
cyanidin-3-glu[a]	*Flavanols*
delphinidin-3-acetylglucoside[a]	catechin [a, b, c]
malvidin-3-acetylgluc[a]	epicatechin [a, b, c]
delphinidin-3-p-coumaroylglucoside[a]	procyanidin dimer 1 [c]
cyanidin-3-p-coumaroylglucoside[a]	procyanidin dimer 2 [c]
petunidin-3-p-coumaroylglucoside[a]	procyanidin dimer 3 [c]
peonidin-3-p-coumaroylglucoside[a]	procyanidin trimer [c]
malvidin-3-p-coumaroylglucoside[a]	procyanidin tetramer [c]
vitisin-A[a]	procyanidin B2 [a, b]
Ac-vitisin-A[a]	procyanidin B1 [a, b]
p-Cm-vitisin-A[a]	gallocatechin [b]
vitisin-B[a]	
Ac-vitisin-B[a]	*Stilbenes*
p-Cm-vitisin-B[a]	trans-piceid [a, b]
10-carboxy-pyranoPn-3-glc[a]	trans-resveratrol [a]
10-catechin-pyranoMv-3-glc[a]	cis-piceid [a]
10-epicatechin-pyranoMv-3-glc[a]	cis-resveratrol [a]
10-(procyanidin dimer)-pyranoMv-3-glc[a]	
10-(procyanidin dimer)-pyranoMv-3-acetylglc[a]	*Stilbenoids*
10-DHP-pymv-3-glc(pinotin A)[a]	cis-resveratrol-3-gluc [c]
10-DHP-pymv-3-acglc[a]	trans-resveratrol-3-0-gluc [c]
10-DHP-pymv-3-cmglc[a]	
10-MHP-pymv-3-glc[a]	*Hydroxybenzoic acids*
10-MHP-pymv-3-acglc[a]	gallic acid [a]
10-MHP-pymv-3-cmglc[a]	syringic acid [b]
(epi)catechin-ethyl-Mv-3-p-coumaroylglc[a]	Hx-es-va, hexose ester of vanillic acid [b]
	Hx-es-pc-a, hexose ester of protocatechuic acid [b]

[a]Ivanova-Petropulos et al. (2015)
[b]Jiang & Zhang (2012)
[c]Rusjan & Mikulic-Petkovsek (2017)

2.2.3.3 *Colour index*

The colour of wines can be quantified using the colour index. The colour of merlot wine is a product of the colour produced by different phenolic compounds dominated by the anthocyanins. Most classes of polyphenols are colourless, except flavonols and chalcones which are yellow pigments, and anthocyanins which overpowers the yellow pigments with red to blue colouration (Cheynier et al., 2010).

2.2.3.4 *Oenological properties*

2.2.3.4.1 pH

Measurement of the pH of wine is necessary because pH influences the microbial growth (useful and pathogenic), taste properties, clarity and colour of wine. Red to purple colouration by anthocyanins only occur at acidic pH (<7). Merlot wines have a typical pH range of 3.2 to 3.6 (González-Neves, Favre, Piccardo, & Gil, 2016).

2.2.3.4.2 Titratable Acidity (TA)

The acids in grape wine and must is mainly tartaric acid, with malic acid, citric acid and succinic acid. Acids in determine pH (Bandici, Vicaş, Teuşdea, Bandici, & Popa, 2017). Zamora, Goldner, and Galmarini (2006) demonstrated the stronger suppressing effect of tartaric acid over the sweetness of fructose in wine samples.

2.2.3.4.3 Total soluble solids (TSS)

TSS is a measure of sugars, mainly glucose and fructose, in grape must and wines. It indicates the maturity of the grapes where alcohol potential can be determined. Merlot grapes are harvested at around 19 -21°Brix (Panceri et al., 2017; Rösti et al., 2018). In wine, TSS readings are corrected to account for the presence of alcohol and are converted into % glucose.

2.2.4 Health related compounds in Merlot wine

Red wines has been associated with the French paradox in 1992, where the wine consuming French population has low incidence of cardiovascular diseases. Studies have shown that the polyphenols present in red wines have high antioxidant activities, which is necessary to protect cells from free radicals that can cause accelerated ageing, destruction and irreversible malfunctions (Saint-Cricq De Gaulejac, Glories, & Vivas, 1999). Clinical trials and in vitro

studies proved the prebiotic benefits (Queipo-Ortuño et al., 2012), lowered risk to atherosclerosis (Chiva-Blanch et al., 2012), and beneficial cardiovascular effects (Wallerath, Poleo, Li, & Förstermann, 2003) of red wine.

Studies on merlot wine showed high antioxidant activity strongly correlated with total phenolics content (Girelli, Mele, Salvagni, & Tarola, 2015). In a study by Jiang and Zhang (2012), the amounts of certain phenolic compounds are significanty correlated with the antioxidant capacity of merlot wine.

Leong, Burritt, and Oey (2016) and Kostadinović et al. (2012) demonstrated the effect of maceration towards increased extraction of phenolic compounds and the increased antioxidant activity and bioprotective capacity (against stress). Longer maceration time (Kostadinović et al., 2012) and PEF treatment (Leong, Burritt, et al., 2016) increased extraction of stilbenes and anthocyanins, respectively.

2.3 Pulsed electric fields processing (PEF)

Pulsed electric fields treatment have shown increased extraction of plant metabolites. It is an emerging food processing technology where intermittent high voltage electric pulses is applied within microseconds to milliseconds across the food product placed between two conducting electrodes (Luengo, Alvarez & Raso, 2015). The resulting electric field causes electroporation of the cell membrane resulting to the release of valuable plant metabolites and cell contents through the pores formed; thus, hastening maceration process in winemaking (Luengo *et al., 2015*). Since PEF is a non-thermal processing technology, heat sensitive polyphenols and aroma compounds are better preserved. Studies have been conducted on diverse grape varieties to produce wine (Delsart et al., 2014; Leong, Burritt, et al., 2016; Puértolas et al., 2009).

This section explains the mechanism of cell electroporation, the parameters in controlling pulsed electric fields treatment, and the application of PEF treatment in winemaking.

2.3.1 Mechanism of electroporation on cells

Electroporation of cells is the formation of pores on the cell membrane caused by the application of short high voltage electric pulses (Donsì, Ferrari, & Pataro, 2010). This is achieved by (1) generating transmembrane potential across the cell membrane; (2) formation of pores on the cell membranes; (3) increase in size and number of pores formed; and (4) the reversible or irreversible electroporation which depends on the intensity of external field strength (Oey et al., 2016).

Opposite sides of the cell membrane has free negative and positive (ions) charges. This results to a voltage called the membrane potential. The difference between the intracellular and extracellular potentials is the initial transmembrane potential. Upon application of PEF into the cell, the electric field accumulates the charges on both sides of the membrane increasing the transmembrane potential (top left of **Fig. 2.2**) (Barsotti & Cheftel, 1999).

Consequently, the heightened attraction of opposite charges inside and outside the cell membrane results to membrane thinning (middle left **Fig. 2.2**). Pore formation is initiated. Closer proximity of the charges from both sides of the membrane, further increase the attraction of opposite charges until this electrocompression exceeds the elastic resistance of the membrane. Larger and more pores are formed (bottom left **Fig. 2.2**) (Barsotti & Cheftel, 1999; Oey et al., 2016).

When the transmembrane potential reaches a critical value E_{crit} (termed rupture potential), the cell membrane is irreversibly electroporated. Potentials lower than the rupture potential results to reversible electroporation where the cells are able to reseal the pores (Barsotti & Cheftel, 1999).

Because of the design of PEF technology (*section 2.3.2*), the intensity of the electric field can be manipulated to produce the desirable extent of electroporation. Electroporation of the cell membrane can be reversible where the cell can recover membrane integrity or irreversible where cell membrane is permanently damaged leading to cell death. The permanence of the pores depends upon the (a) intensity of the PEF treatment which can be controlled by adjusting the external electric field, single pulse duration, and treatment time;

and the (b) cell characteristics such as size, shape and its orientation in the electric field (Raso et al., 2016b).

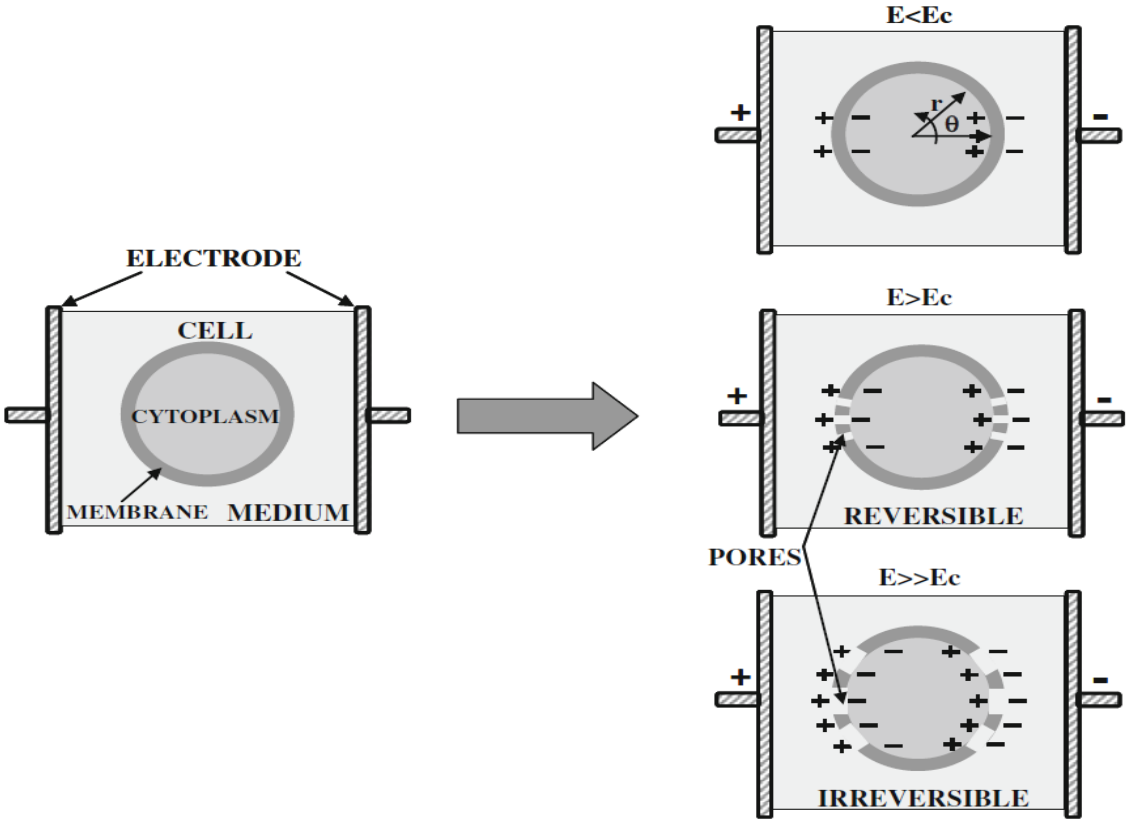

Figure 2.2 Electroporation of a living cell. Lifted from (Donsì et al., 2010).

2.3.2 System components of PEF technology

Pulsed electric fields equipment basically consists of high voltage source, capacitor bank, switch and the treatment chamber. These components work together in delivering a large flux of electrical current towards food in the treatment chamber to generate a high voltage pulsed electric field within the food sample in very short period of time (µs) (Leadley & Williams, 2006).

The high voltage source generates and supplies the electrical energy at the defined intensity, shape, and duration. In operation, the capacitor bank is slowly charged with the electrical energy from the high voltage source, storing energy temporarily. Through the high voltage switch, the capacitor bank delivers the energy in short period of time (µs) towards the electrodes in the treatment chamber and, immediately into the food sample also contained in the chamber. The switch must control and resist the outflow of stored energy from the

capacitor to the electrodes at a required repetition rate, and the backflow of electrical current in a certain intensity caused by the food sample's electrical resistivity (Barbosa-Cánovas & Altunakar, 2006; Barsotti, Merle, & Cheftel, 1999; Leadley & Williams, 2006).

Because the chamber contains the food sample and the released high voltage energy, it must be designed to reduce electrochemical shock and keep the treated food sample inside during pulsing. Chamber designs are being developed to produce uniform treatment across the sample and prevent dielectric breakdown of food. Dielectric breakdown is caused by the application of electric fields higher than the electric field strength of food product producing a spark that damages electrode surfaces. The spark form pits, arching and increased pressure to the electrode surfaces which leads to chamber explosions and evolution of gas bubbles which disrupts the conductivity inside the chamber (Barsotti et al., 1999; Leadley & Williams, 2006).

2.3.3 Main processing parameters influencing PEF

Pulsed electric field treatment is set up according to the desired effect on the target cells in the food samples. For maceration (winemaking) purposes, effective electroporation to release polyphenols and aroma compounds and efficient use of energy are targeted. To achieve optimum operating conditions, processing parameters must be established. These includes the electric field strength (E), pulse geometry/pulse shape, treatment time (t), specific energy (W) and pulse frequency (n), treatment temperature, electrical resistivity (ρ) and conductivity (σ) of food sample.

2.3.3.1 *Electric field strength,* **E**

The electric field strength E determines the extent of electroporation. It is the voltage discharged into the chamber U in kV unit divided by the distance between the two electrodes d in cm (Eq. 1) (Buckow, Schroeder, Berres, Baumann, & Knoerzer, 2010).

$$E = {}^{U}/_{d} \qquad \textit{(Eq. 1)}$$

Electric field strength ranges have been recommended to achieve different levels of electroporation: (i) 15–40 kV/cm inactivates and kills microorganisms; (ii) 1.0–3.0 kV/cm causes irreversible pore formation of cells in plant or animal tissues; and (iii) 0.5–1.5 kV/cm stimulates cells and induces stress responses, electroporation is reversible (Raso et al., 2016b).

2.3.3.2 *Pulse geometry/pulse shape*

Pulse geometry or pulse shape indicate the manner of voltage application into the food sample. Pulses can be unipolar or bipolar, and square wave or exponential. Unipolar pulses retains positive charge (no reversal of polarity in the field). Bipolar pulses is achieved by instant reversal of charges which rapidly reverses the electric field orientation, thought to create additional stress to the cell membrane, hastening electroporation (Leadley & Williams, 2006). Square wave pulses applies constant voltage intensity at the duration of the pulse indicated as the pulse width (**Fig. 2.3**). In exponential pulse, the voltage applied decreases exponentially upon delivery of the set voltage. Its pulse width indicates the time the voltage decreases to 37% of its peak value. The square wave form results to higher electroporation due to longer pulse duration (Barbosa-Cánovas & Altunakar, 2006).

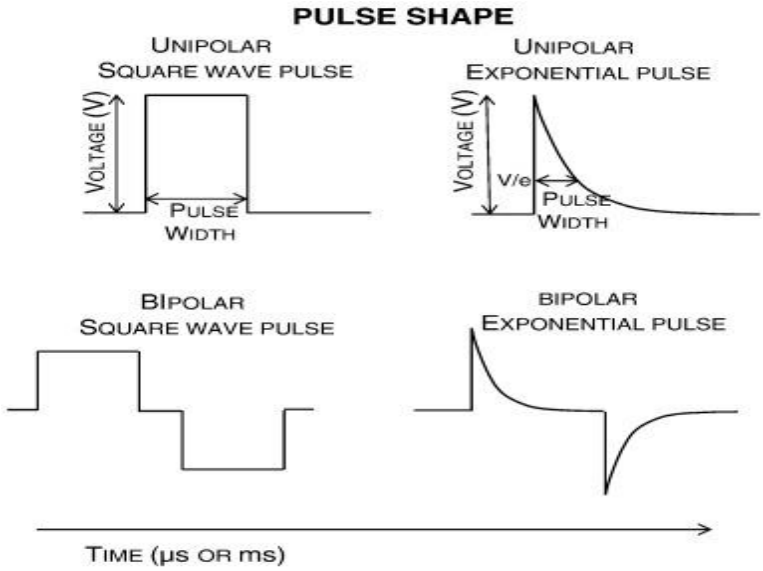

Figure 2.3 Pulse shape commonly used in PEF treatments (Raso et al., 2016).

2.3.3.3 *Treatment time (t), Specific energy (W), and Pulse frequency (f)*

Treatment time is the total time of voltage application towards sample, calculated by multiplying the number of pulses (n) by the pulse width (τ) (Eq. 2) (Raso et al., 2016b).

$$t = n*\tau \qquad (Eq.\ 2)$$

Specific energy (W) is the electrical energy received by the sample from a pulse, taking into account the electrical properties (section 2.3.5) of the food sample and the actual pulse shape which may deviate from the ideal square or exponential waveforms. It is expressed as kJ/kg/pulse and can be computed according to eq. 3.

$$W = \frac{1}{m}\int_0^{\infty} \frac{U(t)^2}{R} dt = \frac{1}{m}\int_0^{\infty} U(t) * l(t) dt \qquad (Eq.\ 3)$$

where *m* is the mass of treated sample, *U(t)* is the voltage across the chamber load and *l(t)* is the current through the chamber load at time *t*.

Pulse frequency (*f*) refers to the repetition rate expressed as the number of pulses applied per second (Hz). Pulse frequency is an important parameter in controlling the amount of electrical energy applied per unit time (Raso et al., 2016b).

2.3.3.4 *Treatment temperature*

Generally, higher initial temperature of samples enhances the degree of electroporation due to the tendency of the biological cell membranes to become more fluid which increases conductivity of the membrane. PEF treatment raises the sample temperature, as well, when electrical energy applied dissipates throughout the sample (Raso et al., 2016b). Hence, PEF treatment should be optimized according to the heat sensitivity of the samples.

2.3.3.5 *Electrical resistivity (ρ) and conductivity (σ) of food sample*

The extent of electroporation by PEF treatment is highly influenced by the intrinsic electrical properties, such as resistivity and conductivity, of the food sample. Electrical resistivity (ρ) refers to the resistance of the sample against the flow of electric current (delivered in PEF treatment). Conductivity (σ) is the opposite and reciprocal in value of resistivity (Barsotti et al., 1999).

These properties of the food samples might vary depending on the phase of the food. Electric current flow through liquids of high conductivity, missing solids of high resistivity (Barsotti et al., 1999). In the case of grapes in winemaking, destemming and crushing prior to PEF treatment aids in the conduction of electricity through the released juices. Further, electroporation of cells and the diffusion of ions from cells further increase conductivity of the sample (Raso et al., 2016b).

2.3.4 Pulsed electric fields in Merlot winemaking

Studies on the optimization of pulsed electric fields processing parameters in Merlot winemaking is limited (Delsart et al., 2012; Leong, Burritt, et al., 2016; Puértolas, López, et al., 2010). As shown in **Table 2.7**, studies done focused on the release of anthocyanins, a general quantification of released phenolic compounds, and their correlation with colour intensity and antioxidant capacity. It can be concluded from the studies that optimum processing parameters may vary between different cultivars and that high intensity PEF treatment, despite releasing more phenolic compounds, does not necessarily produce wine of improved organoleptic characteristics. Therefore, a more holistic approach to optimizing PEF treatment parameters that takes into account the complexity of wine beyond anthocyanins and colour intensity is needed.

2.4 Metabolomic fingerprinting of Merlot wines from PEF-treated grapes

Wine is a complex system composed of hundreds of compounds intricately interacting with each other. However, most of the studies done for advancing wine technology and enhancing quality are based on empirical evidence and is focused on reductionist approach where compounds are singled out of the food matrix (Cozzolino, 2016). For this reason, research in improving wine quality and solving winemaking problems should be addressed in a holistic and integrative approach called metabolomics (Cozzolino, 2016).

Metabolomics is the identification and quantification of small molecule (<1500 Da) metabolites present in a given biological system (Díaz et al., 2016; Wishart, 2008). In wine science, this system pertains to the wine and its collection of compounds is called the wine metabolome.

To identify and quantify all occurring compounds, measurement of the sample through synergistic and complementary analytical platforms able to accurately separate and identify small molecules is necessary (Cozzolino, 2016; Wishart, 2008). These analytical platforms include, but not limited to, chromatographic technology (i.e. ultra-high pressure liquid chromatography such as UPLC and HPLC for rapid compounds separation) in tandem with spectral technology (i.e. mass spectrometry (MS) instruments for precise mass determination) (Wishart, 2008). Equally important are the development of software programs that process these chromatographic and spectral data, as well as the compilation

Table 2.7. Optimization studies on pulsed electric fields treatment of merlot grapes prior to maceration

Parameters optimized	Processing parameters	Effect on macerated grapes or wine qualities	Conclusion on optimized parameters	References
Specific energy input or pulse number and maceration time	d = 0, 2, 4, 8 and 14 days, E = 1.5, $N_{PEF\ low}$ = 243, $N_{PEF\ high}$ = 1033, t = n.d., P = square, f = 50, τ = 20, $W_{PEF\ low}$ = 14.68, $W_{PEF\ high}$= 69.76, batch	• accelerated release of anthocyanins • increased amount of anthocyanins extracted • increased colour intensity • higher antioxidant capacity correlated with increased amount of anthocyanins	Anthocyanins and total phenolic compounds extraction; colour intensity; antioxidant capacity: $W_{PEF\ high} > W_{PEF\ low}$	(Leong, Burritt, & Oey, 2016)
Electric field strength	E = 0, 2, 5, and 7, N = 50, t = 0.15ms, P = square, f = 122, τ = 3, W = 3.67, continuous	• increased extraction of phenols and anthocyanins	Anthocyanins and total phenolic compounds extraction $E_{merlot} > E_{syrah} = E_{cabernet\ sauvignon}$	(Puértolas, López, Saldaña, Álvarez, & Raso, 2010)
Electric field strength and pulse width	E = 0.5 – 0.7, N = 1000, t = 100ms, P = square, f = n.d, τ = 40 and 100, W = n.d., batch	• increased colour intensity in grape must, but no significant difference between wines (7 months old) • increased anthocyanins and tannin	*High* E_{merlot}, *short* τ = *highest tasting score* *Low* E_{merlot}, *long* τ = *lower tasting score* *High* E_{merlot}, *long* τ = *lowest tasting score*	(Delsart et al., 2012)

Abbrevations: t, treatment time; T, application temperature (°C); E, electric field strength (kV/cm);N, pulse number; P, pulse shape (exponential decay or square waveform); f, pulse frequency (Hz); τ, pulse width (μs); W, specific energy input (kJ/kg); batch or continuous mode of treatment. n.d., no data available. (+) increase, (-) decrease or (=) no significant difference in concentration in comparison with the traditionally macerated samples.

of electronic databases containing necessary chromatographic and spectral information to identify the compounds detected in the metabolome (Wishart, 2008).

Examining a metabolome can be done through targeted and untargeted analysis. Targeted analyses detect and precisely quantify a selected pre-defined group/s of compounds. Targeted analysis is limited by the requirement of standard solutions of the compounds of interest. At present, few purified standards of biological metabolites are available (Cambiaghi, Ferrario, & Masseroli, 2017). On the other hand, untargeted analyses, also referred to as metabolite fingerprinting, is a non-discriminatory detection of the compounds attempting to capture all metabolites present. Although this does not precisely quantify, it gives relative quantification of metabolites. However, despite advances in measurement technologies, a bias of detection towards the most abundant compound in the sample is imminent. Through untargeted analysis, characterization of the metabolic profile targets to distinguish discriminatory feature or marker of the metabolome which may be provided but may or may not be identified (Díaz et al., 2016).

Metabolomics lead to the production of large amounts of data from analyses. Comprehensive evaluation of these data sets require a specialized data analysis such as chemometrics where mathematical and statistical methods are used extract meaningful information about possible relations or differences between the different group of samples using the data produced. The information extracted is related to biochemical causes, chemical processes, and, in food sample, organoleptic properties (Krakowska, Custers, Deconinck, & Daszykowski, 2016; Reinholds, Bartkevics, Silvis, van Ruth, & Esslinger, 2015).

However, metabolomics and chemometrics face constraints in terms of the validity of data gathered, comparison of the data generated from other analytical platforms of metabolite separation and detection, and the requirement of rigorous control of the integrity of the data. The insufficient information needed to identify metabolites due to incomplete databases and the limited understanding on many molecular processes that may occur in the sample matrix studied pose as a hindrance (Cambiaghi et al., 2017; Cozzolino, 2016).

Despite these limitations, use of metabolomics and chemometrics has produced information for wine quality control and authentication (Amargianitaki & Spyros, 2017), specific health effects of wine (Sham et al., 2017; Urpi-Sarda et al., 2015; van Dorsten et al., 2010), and wine quality measurement and characterization (**Table 2.8**).

Table 2.8 presents research questions solved by successfully identifying metabolites which can strongly discrimate between wines of the same variety but different planting and winemaking methods. To accomplish this, volatile and non-volatile fingerprinting of juices and wines coupled with a combination of multivariate data analyses seem necessary. These steps in metabolomic analysis is further detailed in the following section.

2.4.1 Steps of fingerprinting approach

The process steps of metabolomics analysis starts with sample preparation, followed by extraction of the metabolites or compounds from the sample matrix, then, separation and detection of the metabolites. Lastly, the data gathered is processed to extract meaningful information (Cevallos-Cevallos, Reyes-De-Corcuera, Etxeberria, Danyluk, & Rodrick, 2009).

2.4.1.1 *Sample preparation*

Optimizing sample preparation aims to reduce experimental error and to optimally extract the compounds from the sample metabolome while ensuring reproducibility and representativeness of the sample (Krastanov, 2010). Pretreatments aims to homogenize texture, structure, viscosity, immiscible phases, hygroscopicity, and hydrophobicity of the samples (Lichon, 1992). Homogeneous samples such as wine may only need concentration or dilution while avoiding overloading (beyond quantifiable amounts) or nondetection (below detection threshold). In untargeted analysis, to reduce discrimination of compounds, minimal sample preparation is preferred (Antignac et al., 2011). Nonetheless, if prior knowledge suggest a particular fraction of the metabolome contains the most useful information, a relatively selective sample preparation can be done for targeted analysis (Antignac et al., 2011).

Table 2.8. Application of metabolomics and chemometrics in wines

Sample	Question/variable	Separation and detection method	Statistical analysis	Conclusion	References
Apulia Negroamaro red wines	Winemaking technologies: ultrasounds (U), cryomaceration (C), traditional (T); Soil management practices: soil tillage (ST), cover crop (CC); vine training systems: monolateral (M) or bilateral (B)	NMR spectroscopy (non-volatile fraction)	Principal Component Analysis, PCA, and Orthogonal Partial Least Square Discriminant Analysis, OPLS-DA	higher glycerol and aromatic compounds content of wines from CC than from ST	(De Pascali et al., 2014)
Sauvignon blanc juices	biogenesis of varietal thiols is not correlated to putative respective precursors	Metabolic profile GC/MS and NMR spectroscopy (volatile and nonvolatile fractions)	Principal Component Analysis (PCA), correlation analysis, ANOVA, *t* tests and hierarchical clustering analysis	24 metabolites greatly impacts varietal thiols evolution	(Pinu et al., 2014)
Pinot noir wines	Wine yard sites: high site-typicity – TG, PT, LG; low site-typicity (IN, F, BR); Vintage: 2012, 2013; Winemaking process: fermentation, barrel aging	SPE -GC/MS (Volatile fraction)	parallel factor analysis (PARAFAC2); principal component analysis (PCA)	4 metabolites discriminated the vineyard sites independent of vintage	(Schueuermann, Khakimov, Engelsen, Bremer, & Silcock, 2016)
Sangiovese wine	Optimum storage conditions in a wine cellar vs typical domestic storage	HILIC (hydrophilic interaction) LC–MS (Non-volatile fraction)	principal component analysis (PCA); Orthogonal partial least-squares discriminant analysis(OPLS-DA)	4-amino-heptanoic acid was a marker for wine sub-optimum storage conditions (domestic storage)	(Arapitsas et al., 2016)

2.4.1.2 *Extraction*

An extraction step maximizes the amount and concentration of the compounds of interest. This relies on the prior knowledge on the properties of compounds present in the sample, such as solubility to solvents which may be in pure or mixed forms, or affinity to polar or non-polar fibers. Samples containing compounds of opposing properties might require sequential extraction. For untargeted analysis, an extraction techniques must be compared in terms of the number of different compounds extracted (Cevallos-Cevallos et al., 2009).

In this study, headspace solid phase microextraction (HS-SPME) technique will be used to extract volatile compounds using a bipolar fiber to reduce bias between polar and nonpolar compounds.

2.4.1.3 *Separation and detection methods*

Metabolites or compounds are analysed individually requiring separation from the rest of the metabolome. Separation techniques are commonly chromatographic technologies that relies on the differential affinities of compounds towards the material of the equipment, usually attached in a column where the sample passes through. These techniques include high performance (HP) and ultra-performance (UP) forms of liquid chromatography (LC), gas chromatography (GC), and capillary electrophoresis (CE). Separation techniques are coupled with detection techniques such as mass spectrometry (MS), nuclear magnetic resonance (NMR), and near infrared spectrometry (NIR) (Cevallos-Cevallos et al., 2009).

Separation and detection methods used should be robust, precise and provide reproducible and representative data of the sample. Metabolomics may combine data generated from different analytical methods required due to the difference in the optimum extraction, separation and detection method of a metabolome fraction. (Cevallos-Cevallos et al., 2009; Cozzolino, 2016; Wishart, 2008). This combination of analytical platforms has been done on Sauvignon Blanc juice (**Table 2.8**).

2.4.1.4 *Data analysis*

Before statistical analysis, the bulk of data gathered from metabolomics is pre-processed to remove measurement noise (or baseline) and correct instrumental deviations on retention/migration times. This is done through deconvolution, filtering, feature detection, alignment and normalization (Cevallos-Cevallos et al., 2009; Katajamaa & Oresic, 2007). Normalization can be done using an internal standard (a compound that is non-existent in the matabolome) to reduce experimental error.

Because metabolomic analyses gathers measurements of multiple variables (such as shown in **Table 2.8**), the data gathered will be multivariate in nature. In multivariate data statistical analysis, redundant data is removed and variation not relating to analytical signal is reduced. Multivariate methods commonly used include principal components analysis (PCA) and partial least squares regression (PLS). PCA is used to group samples according to a created factor that gives the highest variance among the groups of data. PLS is commonly applied on data analysed to create models such as predictive metabolomics studies for sensory evaluation (Cevallos-Cevallos et al., 2009; Krastanov, 2010) Using appropriate statistical tool, the large amount of data from metabolomics can be translated into a distinguished biomarkers that discriminates a metabolome from the others.

2.4.2 Metabolomic fingerprinting of wines and grape juice

Application of metabolomics and chemometrics in wine studies is still very limited. **Table 2.8** shows single studies in wine evaluating and determining the variable (from multiple variables) with the most important impact in the wine/grape metabolome.

2.5 Conclusion

Pulsed electric fields treatment of grapes prior to maceration has been proven to extract more polyphenols and aroma compounds. These are important metabolites that dictate the organoleptic quality and health effects of Merlot wine. However, the significant difference between treated and untreated grapes is not well verified after fermentation and after ageing, when the extracted metabolites have already evolved. Further, PEF processing parameters are not yet optimized based on volatile profile in combination with the polyphenols composition and their interactions in the whole wine matrix – a more holistic and integrative analysis of metabolomics approach. To address this knowledge gap, this study will elucidate

on how varying intensities of PEF treatment affect the grape must and wine samples at different winemaking process points (maceration, alcoholic fermentation, malolactic fermentation, and bottles storage) through fingerprinting using two analytical platforms – targeted profiling of phenolic compounds (HPLC-DAD), colour, and oenological properties, and untargeted fingerprinting of volatile composition (HS-SPME-GCMS).

CHAPTER 3

FEASIBILITY OF USING INTEGRATED FINGERPRINTING, PROFILING, AND CHEMOMETRICS APPROACH TO UNDERSTAND (BIO) CHEMICAL CHANGES THROUGHOUT COMMERCIAL RED WINEMAKING: CASE STUDY ON MERLOT

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3.1 Introduction

The colour, aroma, taste, and mouthfeel of red wine are determined by the concentration and composition of phenolic and volatile compounds. Phenolic compounds in red wine include flavonoids such as anthocyanin, flavonol, flavanol, and flavanonol, and non-flavonoids including stilbene, hydroxycinnamic acid, and hydroxybenzoic acid (Ginjom, D'Arcy, Caffin, & Gidley, 2011). While volatile compounds in red wine can be classified based on differences in their chemical structure such as ester, higher alcohol, terpene, fatty acid, aldehyde, ketone, or norisoprenoids (Arcari, Caliari, Sganzerla, & Godoy, 2017), their composition are highly influenced by the grape variety, fermentation and aging process.
In red wines, simultaneous maceration and alcoholic fermentation (MAF) majorly responsible for extracting phenolic and aroma precursors along with other plant metabolites from the grape berry cells (Cheynier, Schneider, Salmon, & Fulcrand, 2010). Ethanol produced during MAF due the yeast action further facilitates the extraction of these compounds (Canals, Llaudy, Valls, Canals, & Zamora, 2005). Most phenolic and aroma precursors extracted are in their glycosylated forms, which depresses the volatility of aroma compounds (Fontes, Gerós, & Delrot, 2011; Hjelmeland & Ebeler, 2015). During MAF, enzymatic and chemical hydrolysis of glucosides could take place that releases bound phenolic and volatile aroma compounds. Additionally, oxidation reactions, complex formations, or even re-glycosylation can occur concurrently at this stage (Lingua, Fabani, Wunderlin, & Baroni, 2016). The yeast can further affect the phenolic and volatile profiles of the must through synthesis of new compounds such as alcohols and esters by its metabolism, and through the loss of some compounds by adsorption on the yeast cell (Morata et al., 2003).

Malolactic fermentation (MLF) usually takes place right after MAF, where lactic acid bacteria (LAB) is utilised to convert harsh malic acid into the mellower lactic acid by decarboxylation (Bartowsky, 2014). Phenolic and volatile profiles and other oenological properties are modified through mechanisms including further de-glycosylation of bound volatile compounds, adsorption of compounds on the bacterial cells, bacterial consumption of leftover sugar, amino acids, nitrogen, and other nutrient compounds producing new volatile and non-volatile end products, and autolysis of the microbial cells releasing flavour-impacting cell metabolites (Bartowsky, 2014; Malherbe, Tredoux, Nieuwoudt, & du Toit, 2012). It has been shown that MLF process predominantly responsible for increasing the

free forms of hydroxycinnamic acids (Cabrita et al., 2008; Hernández, Estrella, Carlavilla, Martín-Álvarez, & Moreno-Arribas, 2006), as well as facilitating the polymerisation of tannins and anthocyanins, forming stable colour complexes (Bartowsky, 2014).

Altogether, winemaking involves a complex pool of thousands of phenolic compounds and aroma precursors, enzymes, yeasts, and lactic acid bacteria interacting with each other. However, current literature explains the evolution of phenolic and volatile compounds occurring during winemaking independently; as separate studies for different winemaking stages. Most studies focused on the changes of few *a priori* selected class of phenolic compounds during the winemaking process (Cejudo-Bastante, Vicario, Guillén, Hermosín-Gutiérrez, & Pérez-Coello, 2015; Devi, Archana, Bhavya, & Anu-Appaiah, 2017; Gil-Muñoz, Gómez-Plaza, Martínez, & López-Roca, 1999; Ginjom et al., 2011). With respect to volatile compounds, a meta-analysis conducted by Ilc et al. (2016) has revealed a close relationship between the effect of MAF and MLF on aroma-relevant compounds in wines. It is important to note that the approaches followed in most previous studies targeted solely on selected few attributes and neglected the interaction between the phenolic, volatile and other oenological wine properties. Therefore, there is a need for a comprehensive multiplatform approach to increase insight into the complex and dynamic (bio)-chemical changes during winemaking.

To address this gap, for the first time in literature, this study systematically integrated untargeted fingerprinting analysis and targeted profiling of grape musts, fermenting juice and wines. The untargeted fingerprinting of the volatile fraction was performed through HS-SPME-GC-MS, and the targeted profiling of phenolic compounds was carried out through HPLC-DAD. Since the data obtained from such multiplatform analytical approach can be overwhelming, fingerprinting and profiling data were fused and then analysed using advanced chemometrics methods (e.g. partial least squares discriminant analysis) to unravel the complex reactions and identify discriminant markers for each winemaking stage. These markers can then be related to changes in the (bio)-chemical and chemical processes, and organoleptic properties during each winemaking stage. Merlot (*Vitis vinifera* L.) is one of the most widely planted red grape cultivars intended for wine production and yet the evolution of volatiles and phenolics over the course of winemaking process is still not well understood. Therefore, the objective of the present study was to understand how commercial red winemaking process, encompassing from pre-maceration (PM), maceration-alcoholic

fermentation (MAF), up to completion of malolactic fermentation (MLF), influence the oenological attributes (soluble solids, pH, titratable acidity and colour properties), headspace volatiles and phenolic composition of Merlot.

3.2 Materials and methods

3.2.1 Merlot grape berries

Merlot grapes (*Vitis vinifera L.*) harvested from Hawke's Bay region (39 °South, 176 °East; North Island, New Zealand) at the end of March 2016 were used in this study. The physicochemical properties of grapes at harvest were 18.00 – 18.60 °Brix, titratable acidity of 5.11-5.19 g tartaric acid/L, and pH of 3.45-3.48. The grapes were transported overnight by a refrigerated truck to a winery and stored at 5°C (<48 h) until further processing. The grapes were destemmed through a continuous Wottle A1 grape destemmer (Anton, Poysdorf, Austria).

3.2.2 Merlot commercial winemaking and sampling

Immediately after destemming and crushing, the grape must was pumped into 500 L open top fermenters. Aliquots of must samples (consisting of juice, skins and seeds) were then sampled immediately (thereafter referred as pre-maceration or "PM" sample) whereby the grape musts were strained through a kitchen grade wire mesh (1 mm^2) and the filtered juice was stored at -18 °C until further analysis.

Merlot was vinified according to standard practices for production of commercial wines by experienced winemakers. In brief, sulphite (50 ppm in the form of potassium metabisulphite) was added to the must, followed by addition of commercial wine yeasts (*Saccharomyces cerevisiae*) within 24 h to initiate the alcoholic fermentation process. The fermentation occurred between 16.5 and 25.5 °C in 500 kg tanks. This is a temperature-controlled fermentation. The temperature was monitored every 12 h and the temperature was raised according to the must brix. The end of alcoholic fermentation was reached after 7 days when the sugars were almost completely consumed (<2 g/L). Aliquots of fermenting grape musts were also sampled (thereafter referred as maceration-alcoholic fermentation or "MAF" sample) and stored at -18 °C until further analysis. Afterwards, the wine was pressed from the skins/seeds and malolactic bacteria was added. Malolactic fermentation took place in 50 L metal kegs. Then, the wine was racked off the malolactic lees, 30 ppm potassium

metabisulphite was added and stored cool for 5 months at 12-13 ^{o}C. Then, the wine was bottled under inert gas, sealed and stored at 4 ^{o}C until analysis (thereafter referred as malolactic fermentation or "MLF" samples).

3.2.3 Basic oenological parameters of grape juice and wine

To monitor the progress of maceration and fermentation processes, oenological parameters, namely total soluble solids (TSS), pH, and titratable acidity (TA), were determined. TSS and pH were measured using a refractometer (Atago, Tokyo, Japan) and a pH meter (Hannah pH 209, Hannah Instruments, Inc., Woonsocket, USA), respectively. To determine the TA, 5 mL samples were titrated against 0.1 N sodium hydroxide (NaOH, VWR Prolabo, Leicestershire, England) and the volume of NaOH required to reach pH 8.2 ± 0.05 was recorded. TA was expressed as gram of tartaric acid per litre of sample, using Equation 1, where N is the normality of NaOH, T mL of NaOH is the volume of titrant, 75 is a conversion constant for tartaric acid, and 5 mL of sample is the volume of sample used.

Tartaric acid (g/L) = (N NaOH x T mL NaOH x 75)/5 mL of sample *(Eq. 1)*

Measurements of total TSS, pH and TA for each sample was performed in triplicates.

3.2.4 Colour characteristics of grape juice and wine

The colour characteristics of the grape juice and wine samples were measured in triplicates through their colour intensity (CI) index via a UV/vis spectrophotometer, as well as *L**, *a**, *b**, chroma (*C**), and hue (h^o) values using a colourimeter. CI was calculated as the sum of the absorbance of sample at wavelengths 420, 520, and 620 nm. Samples were centrifuged at 9335 *g* for 5 min (IEC Micromax Centrifuge fitted with IEC 851OF Fixed-Angle Rotor, International Equipment Company, Massachusetts, USA) and the supernatant was diluted appropriately prior to spectrophotometric measurement. The samples were analysed using a UV/Vis spectrophotometer (Ultrospec 3300 Pro, Amersham Biosciences, Sweden). Differences in colour attributes between samples were further evaluated using the *L**, *a** and *b** coordinates measured through a HunterLab Colourimeter (MiniScan EZ 4500S, Virginia, United States). Increasing *L** value indicates an increase in lightness. Increasing values of *a** indicates a shift from blue to yellow; while increasing values of *b** signifies a change from green to red. Furthermore, *C** and *h°* were also calculated using Equations (2) and (3).

$$C_{ab} = \sqrt{a^{*2} + b^{*2}} \quad \textit{(Eq. 2)}$$

$$h^{o} = \text{arc tan } (b^*/a^*) \quad \textit{(Eq. 3)}$$

Chroma refers to the colour saturation, which is proportional to the CI. High *C** indicates more vivid colour. Hue specifies the colour through angle degree values in the colour space. Red, yellow, green, and blue hues are represented as angles 0°/360°, 90°, 180°, and 270°, respectively.

3.2.5 Profiling of anthocyanin and phenolic compounds using HPLC-DAD

Reversed-phase high-performance liquid chromatography (HPLC) was used to separate different classes of phenolic compounds present in the grape juice and wine samples. Each sample was gently thawed overnight in a cool room (4 ^{o}C). Then, samples were centrifuged at 9335 *g* for 5 min and the supernatant was filtered through a 0.45 µm pore size cellulose syringe filter. Five microliters (5 µL) of each sample were then injected into the reversed-phase Kinetex C18 column (100 x 4.6 mm i.d., 2.6 µm pore size; Phenomenex), protected with a KrudKatcher ULTRA HPLC in-line filter (0.5 µm depth x 0.10 mm internal diameter, Phenomenex), and maintained at 20 ^{o}C. The mobile phase composed of solvent A: 10% (v/v) formic acid (EMSURE®, Merck, Darmstadt, Germany) in acetonitrile (HiPerSolv CHROMANORM®, VWR International, Pennsylvania, United States); and solvent B: 10% (v/v) formic acid in MilliQ water (Sartorius arium 611UV water purification system, Göttingen, Germany) with flowrate at 1 mL/min on an Agilent 1200 system (Agilent Technologies, Palo Alto, CA, USA). Individual anthocyanin and phenolic compounds were separated under the following elution condition: 0-12.5 min, 96.7% B; 12.5 – 13.5 min, 64.3% B; 13.5 – 14.5 min, 96.7% B; and 14.5 – 20 min, 96.7% B. All classes of phenolic compounds were detected using diode-array detector (DAD) whereby the UV spectra were recorded in the range of 200 and 600 nm. Anthocyanins were observed at 520 nm; flavonols at 360 nm; hydroxycinnamic acids and stilbenes at 325 nm; and flavanols, flavanonols, and hydroxybenzoic acids at 280 nm. The identification of individual anthocyanin and phenolic compounds was performed according to a previous work by Leong, Burritt, et al. (2016) using LC-ESI-MS analysis and by matching retention times with known standards (95% purity), and afterwards quantified through external calibration curve of the respective

authentic standard. All standards were prepared in HPLC grade methanol (LiChrosolv®, Merck, Darmstadt, Germany) prior to column injection. Analysis was performed in six replicates for each sample. Results are expressed as milligram of phenolic per litre of sample.

3.2.6 HS-SPME-GCMS fingerprinting of volatile compounds

3.2.6.1 *Sample preparation and volatile extraction*

Volatile compounds were extracted using headspace-solid phase micro-extraction (HS-SPME), and analysed using the Agilent 6890N GC system connected to an Agilent MSD 5975 VL. Equilibration, extraction and desorption of volatile compounds were automated using an auto sampler. Aiming for an untargeted fingerprinting, the HS-SPME-GC-MS method of analysis was optimised in advance in order to detect a wide range of volatile compounds. The optimisation includes sample dilution, salt-to-sample ratio, SPME fibre incubation and extraction conditions and GC-MS analysis parameters. An SPME fibre with a balanced polarity (50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS)) was chosen due to its ability to capture a wide range of volatile compounds from different chemical classes (Kebede et al., 2013). The SPME fibres were conditioned and regenerated according to the manufacturer's guidelines. Each sample was gently thawed overnight in a cool room (4 ^{o}C). Two millilitres of samples were mixed with 6 mL MilliQ water and saturated with 2.5 g sodium chloride in a headspace glass vial. The vials were tightly capped with PTFE/silicone septum and crimp caps. These were then equilibrated at 40 ^{o}C for 5 min with agitation prior to headspace SPME extraction at 40 ^{o}C during 30 min. Six independent SPME extraction was performed for each sample.

3.2.6.2 *GC-MS procedure*

After volatile extraction, the SPME fibre was inserted into the GC injection port, where the volatile compounds were thermally desorbed at 230 °C during 5 min in a splitless mode. Using helium as the carrier gas, the volatile compounds were separated through the ZB-Wax column (60 m × 0.32 mm inner diameter × 0.5 µm film thickness; Phenomenex) at a constant flow rate of 1 mL/min. The GC oven temperature was initially held at 50 ^{o}C for 5 min, followed by heating at 5 ^{o}C/min to reach 210 ^{o}C, and then further ramped at 10 ^{o}C/min to reach the final 240 ^{o}C and maintained for the next 5 min before cooling to 50 ^{o}C. Electron ionisation (EI mode) at 70 eV was used to obtain the mass spectra with a scanning range of

35 to 400 m/z. The MS ion source and MS quadrupole temperature was set at 230 ^{o}C and 150 ^{o}C, respectively.

3.2.6.3 *Data pre-processing of total ion chromatograms*

GC-MS total ion chromatograms, usually laden with co-eluting peaks, were analysed using an automated mass spectral deconvolution and identification system (AMDIS, version 2.72, 2014) to deconvolute and to extract "pure" component spectra, which aids for a more accurate identification of the peaks using NIST spectral library (NIST14, version 2.2, National Institute of Standards and Technology). The deconvoluted spectra were further analysed by Mass Profiler Professional (MPP) software (Version 14.9.1, 2017, Agilent Technologies) to filter and align peaks, producing the data table containing each peaks quantity level expressed as peak areas. In the present work, three criteria were employed to increase the power of volatile identification: (a) match and reverse match with the NIST library of not less than 90%; (b) comparison of the experimental retention index (RI) with RI according to literature; and (c) matching retention time and spectra with standards for each chemical group detected volatiles.

3.2.7 Data analysis

3.2.7.1 *Univariate statistical analysis of wine oenological properties*

One-way analysis of variance (ANOVA) and Tukey's HSD test at $p < 0.05$ level were used to assess the statistical differences between samples at different stages of winemaking. Measurement of oenological properties were expressed as average ± standard deviation.

3.2.7.2 *Multivariate statistical data analysis (MVDA)*

To better understand the interrelationship between the attributes, the multivariate data analysis (MVDA) was performed in a step-wise approach: (i) MVDA of phenolic profile; (ii) MVDA of volatile fingerprint; and finally, (iii) MVDA combining all measured attributes namely volatile and phenolic compounds, colour, and oenological properties. The MVDA was conducted using SOLO software (Version 8.6, 2018, Eigenvector Research, Wenatchee, WA, USA). The tabulated data were firstly mean-centred and the variables were weighed by their standard deviation to give them equal variance. In the next step, an unsupervised classification of the data with principal component analysis (PCA) was carried out as an exploratory technique to evaluate the data for outliers and groupings. Following that, to study

the changes during the winemaking process step, the multivariate data was further analysed through partial least squares-discriminant analysis (PLS-DA), which is a regression-based supervised classification technique. In this analysis, the optimum number of latent variables (LV) was selected with a criterion to explain the maximum variance/information in the data while maintaining the error to a minimum. Based on the PLS-DA model, bi-plots were constructed to study the evolution during the winemaking stage. Finally, to identify metabolites significantly affected by the winemaking stages (potential markers), variable identification coefficients (VID) were calculated. VID values are the corresponding correlation coefficients between *X*-variables (attributes) and predicted *Y*-variable (winemaking stage). Attributes with an absolute VID value of at least 0.800 were selected as discriminant markers. These markers were then identified (see **Section 3.2.6.3**) and linked to potential reaction pathways during commercial red winemaking.

3.3 Results and discussion

3.3.1 Oenological properties and colour characteristics

Table 3.1 shows the significant changes ($p < 0.05$) in TSS, pH, and TA values of grape samples upon completion for each winemaking stage, namely pre-maceration (PM), maceration-alcoholic fermentation (MAF), and malolactic fermentation (MLF). At PM, it was clear that Merlot grapes used in this study contained an initial high level of fermentable sugars that could be utilised later by yeast. Due to the progression of fermentation processes by yeast cells during MAF, TSS and pH decreased while TA increased. MLF slightly lowered TA and raised pH. The fermentation completed at ABV of 11.25. The obtained TSS, pH, TA, and ABV values in this study were comparable to typical vinification values (Cheynier et al., 2010).

Significant changes ($p < 0.05$) in the colour of fermenting grape juice to wine was observed through the measurement of CI, L^*, a^*, b^*, C^*, and h^o (**Table 3.1**). CI significantly increased after MAF, which coincided with the decrease in L^* and h^o, denoting that the colour of the fermenting musts was more intense, darker, and redder. However, L^* and CI decreased while h^o increased upon the completion of MLF, indicating a darker colour but with a lower intensity compared to samples at MAF. The trend towards a more intense, darker, and redder colour after MAF, and the subsequent decline in colour intensity after MLF has been observed in previous studies on Merlot and other grape varieties (Burns & Osborne, 2013;

Mazza, Fukumoto, Delaquis, Girard, & Ewert, 1999). This is because monomeric anthocyanins and other phenolic compounds responsible for the colour of the must are susceptible for release in a huge amount from the grape skins through the simultaneous maceration and alcoholic fermentation process (MAF) (Canals, Llaudy, Valls, Canals, & Zamora, 2005). On the other hand, it is rather common that the colour stability of anthocyanins is affected by the action of various enzymatic and chemical reactions occurring during MLF (Cheynier et al., 2010).

Table 3.1 Oenological properties and colour expressions of grape juices and wines sampled at pre-maceration (PM), after maceration-alcoholic fermentation (MAF), and after malolactic fermentation (MLF).

	Pre-maceration (PM)	**Maceration-alcoholic fermentation (MAF)**	**Malolactic fermentation (MLF)**
TSS	18.33 ± 0.27^{a}	5.80 ± 0.00^{b}	6.00 ± 0.00^{b}
ABV	-	-	11.25 ± 0.13
pH	3.47 ± 0.01^{a}	3.37 ± 0.01^{c}	3.43 ± 0.02^{b}
TA	5.14 ± 0.03^{c}	9.33 ± 0.05^{a}	6.35 ± 0.11^{b}
CI	1.81 ± 0.13^{c}	10.58 ± 0.23^{a}	7.54 ± 0.37^{b}
L*	18.09 ± 0.10^{a}	7.51 ± 0.11^{b}	3.97 ± 0.06^{c}
a*	27.08 ± 0.28^{a}	21.63 ± 0.11^{b}	18.77 ± 0.11^{c}
b*	15.24 ± 0.22^{a}	2.78 ± 0.08^{c}	4.44 ± 0.08^{b}
C*	31.07 ± 0.34^{a}	21.81 ± 0.11^{b}	19.29 ± 0.11^{c}
***h*°**	0.51 ± 0.00^{a}	0.12 ± 0.00^{c}	0.23 ± 0.00^{b}

Data expressed as mean ± standard deviation (3 replication). Means with the same superscript letters in the same row have no significant difference (Post Hoc Tukey's HSD test, $p < 0.05$). Oenological properties: TSS, total soluble solids (°Brix); SG, specific gravity; ABV, alcohol by volume; TA, titratable acidity (g tartaric acid/L); CI, colour intensity ($A_{420} + A_{520} + A_{620}$); *L**, lightness (100, white; 0, black); *a**, redness (+, redder; -, greener); *b**, yellowness (+, yellower; -, bluer); *C**, *chroma (*+, brighter; -, duller*); h*°, hue (0° and 360°, red; 90°, yellow; 180°, green; 270°, blue).

3.3.2 Phenolic profile

Phenolic compounds in red wines are complex and reactive biomolecules. Their interactions with each other or other molecules throughout winemaking accounts for the resulting wine colour, bitterness, astringency, and antioxidant properties. Visual analyses of the representative HPLC-DAD chromatograms at 520 nm (anthocyanins), 360 nm (flavonols), 325 nm (hydroxycinnamic acids and stilbenes), and 280 nm (hydroxybenzoic acids, flavanol, and flavanonol) reveals differences in composition and peak areas/heights of phenolic compounds brought about by the different vinification processes (**Figure 3.1**). Overall, 36 phenolic compounds were identified and quantified using authentic standards. Substantially,

MAF increased the number and concentration of phenolic compounds followed by reduction, loss, and appearance of some peaks after MLF. This observation is in line with the colour changes discussed in **Section 3.3.1**. The changes in phenolic compounds will be discussed together with other attributes in detail in **Section 3.3.4**.

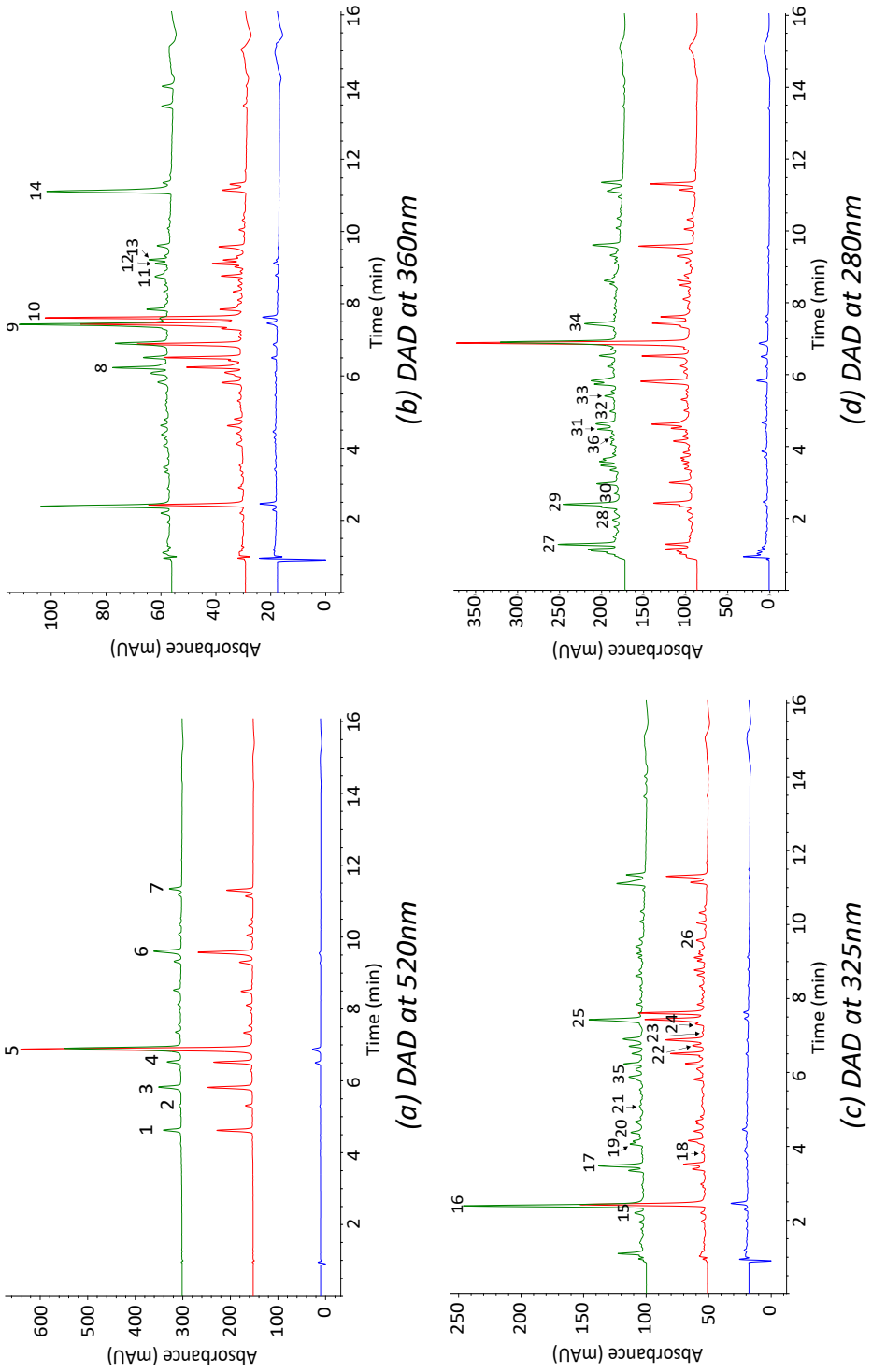

Figure 3.1. Visual representation of the HPLC-DAD chromatograms detected at (a) 520 nm (anthocyanins), (b) 360 nm (flavonols), (c) 325 nm (hydroxycinnamic acids and stilbenes), and (d) 280 nm (hydroxybenzoic acids, flavanol, and flavanonol) for grape must and wine samples collected at pre-maceration, PM (i); maceration-alcoholic fermentation, MAF (ii); and malolactic fermentation, MLF (iii).

3.3.3 Volatile fingerprint

Figure 3.2 shows representative total ion chromatograms obtained using the HS-SPME-GC-MS analysis after each winemaking step (PM, MAF, and MLF). By comparing the chromatograms chronologically, the clear differences in peak areas reveal the progression of Merlot's volatile fraction as a function of the winemaking stages. Average number of volatiles detected by the headspace fingerprinting method ranged from 29 at PM to 62 after MAF and finally 80 compounds after MLF. The volatile compounds detected in this study can be classified into esters, organic acids, alcohols, aldehydes, terpenes, and norisoprenoids chemical groups. As an example, the most abundant peaks in each representative chromatogram are identified on **Figure 3.2**. The volatile compounds detected in this work are in agreement with other studies on Merlot juice and wine (Arcari et al., 2017; Hernandez‐Orte et al., 2014; Panceri et al., 2017; Song et al., 2016; Zhang et al., 2013).

3.3.4 Multivariate data analysis integrating volatiles, phenolic and oenological attributes

3.3.4.1 *Visualisation of the overall change/trend during the transformation of Merlot must to wine*

To better interpret the mutual importance of the selected quality parameters and their link to changes during vinification, the multivariate data analysis (MVDA) was performed in a step-wise approach: (i) MVDA of phenolic profile; (ii) MVDA of volatile fingerprint; and finally, (iii) MVDA combining all measured attributes namely volatile and phenolic compounds, colour, and oenological properties. In all three multivariate models, measured attributes were designated as *X*-variables, while the winemaking stages (PM, MAF, and MLF) were assigned as categorical *Y*-variables. PCA was used first as an exploratory technique to reveal similarities or differences between samples and to detect outliers. No outliers were detected and classes (PM, MAF, and MLF) were clearly separated (results not shown). Next, PLS-DA models were performed using two latent variables (LVs). The first two LVs accounts for 93 to 98% of the *Y*-variance. **Figure 3.3** shows the three bi-plots constructed based on each PLS-DA model. The bi-plots illustrate a clear difference between the winemaking stages for all three data: (a) phenolic profiles; (b) volatile fingerprints; (c) combination of all analysed attributes.

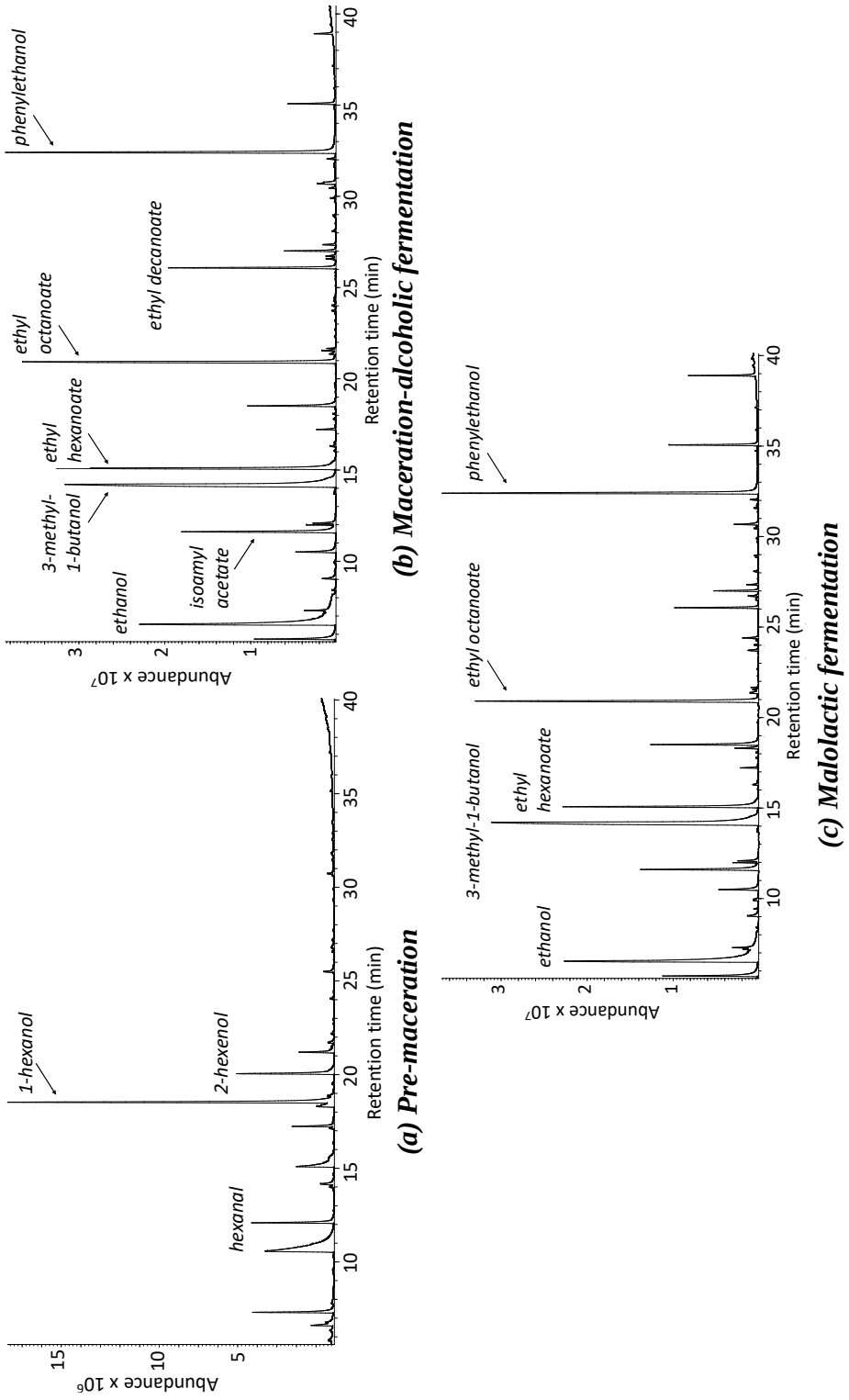

Figure 3.2 Comparison of total ion chromatograms of grape must and wine samples at (a) pre-maceration, PM; (b) after maceration-alcoholic fermentation, MAF; and (c) after malolactic fermentation, MLF, obtained with the headspace SPME-HS-GC-MS fingerprinting procedure.

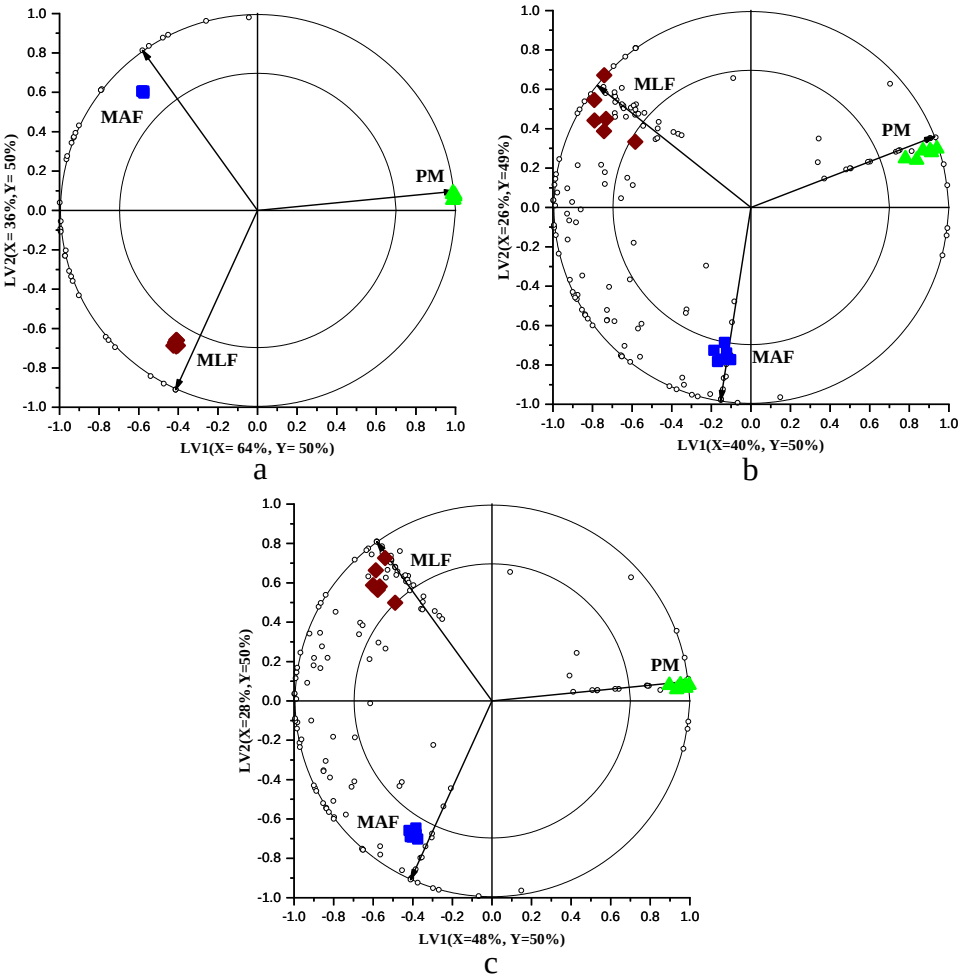

Figure 3.3 PLS-DA bi-plots illustrating the variance between pre-maceration (PM), maceration-alcoholic fermentation (MAF), and malolactic fermentation (MLF) samples based on their (a) phenolic profiles, (b) volatile fingerprint, and (c) combined volatile fingerprin t, phenolic profiles, colour, and basic oenological properties.

Bi-plot is a useful tool to visualise the discriminative power of the measured attributes. The positions of attributes (*X*-variables) on the plot, represented as small open circles, signify their importance. Components have increased discriminative power when they are positioned further away from the centre, particularly between the inner and outer circles on the bi-plot (which respectively mark 70% and 100% correlation coefficient). From **Figure 3.3**, most of the compounds are positioned between the two circles; implying that a large number of these compounds has strong discriminative power (Kebede et al., 2013). Further on the plot, an attribute positioned closer to a certain sample is considered to be positively correlated to that sample, while an attribute farther away in opposite direction is likely negatively correlated to that particular sample (Kebede et al., 2013). Hence, **Figure 3.3** reveals that a significant number of components (deemed important due to location in

between the circles) are negatively correlated with PM. This suggests that PM has a relatively low value or amount of these attributes, and that these attributes seem to accumulate in the subsequent vinification steps (MAF and MLF) where these components are found to be located closer to those samples.

3.3.4.2 *Selection and interpretation of discriminant markers per winemaking stage*

Although a bi-plot is a useful tool to visualize the changes/trend, further selection of discriminant components was performed to determine potential markers driving the separation between winemaking stages. Hence, variable identification (VID) coefficients were calculated based on the PLS-DA model with all attributes combined (**Figure 3.3c**). VID coefficient refers to the correlation coefficient between the *X*-variables and predicted *Y*-variables. A strong positive coefficient value signifies high compound concentration or high attribute value in one winemaking stage compared to the others, and *vice versa*. In the present work, compounds with an absolute VID value higher than 0.800 were identified and listed as discriminant markers (**Table 3.2**). To clearly show the changes in value or concentration, a bar graph was plotted for selected representative markers at each winemaking stage (**Figure 3.4**).

At PM, fifty-one discriminant markers were selected in the unfermented grape musts (Table 2). Ten of the markers were selected with positive VID coefficients, which includes TSS, colour parameters (e.g. *L**), 2-hexen-1-ol, 2-hexen-1-al, acetic acid, and octen-3-ol. The remaining 41 markers (e.g. anthocyanins, hydroxycinnamic acids, fatty acids, alcohols, and a terpene) were all selected with negative VID values, indicating that these compounds were present in significantly (p<0.05) lower amounts at PM and seem to increase along the vinification processes. TSS is among one of the basic oenological properties measured to monitor the progress of vinification, as soluble sugars readily available in the grape must/juice was expected to decrease upon fermentation into alcohol and carbon dioxide (**Figure 3.4**). Additionally, high *L** value at PM indicates lighter colour in the musts due to low concentrations of pigments (i.e. anthocyanins) being extracted from grape skins before maceration commenced (**Figure 3.4**). Both 2-hexen-1-ol and 2-hexen-1-al are commonly associated with vegetal, herbaceous, freshly cut grass aroma in Merlot grape juice and wines. These green odourants are collectively known as the C6 (six-carbon) compounds in which they are formed through a series of enzymatic reactions involving the polyunsaturated fatty

acids accumulated in the grape skins. Pre-fermentation processes including the harvesting, transport, destemming, crushing and pressing favour the release of lipid precursors from the skin and consequently, accelerate the production of these freshness and greenness associated volatiles in the musts (Oliveira, Faria, Sá, Barros, & Araújo, 2006). For this reason, C6 compounds are very often categorised as pre-fermentation related volatiles. While the presence of C6 volatiles at higher levels can lead to undesirable aroma perception in finished wine (Mendez-Costabel et al., 2014), fortunately findings from this work demonstrated that these volatiles initially detected in high level at PM stage experienced dramatic decline after the eventual fermentation processes of MAF and MLF (**Figure 3.4**).

Table 3.3.1 Discriminant volatile, phenolic, and oenological attributes determined based on highest absolute VID coefficients for Merlot grape juices and wines sampled at pre-maceration (PM), maceration-alcoholic fermentation (MAF), and malolactic fermentation (MLF). The compounds and properties are listed in decreasing order of VID coefficients where positive high value pertains high association with the sample and negative high value pertains low association with the sample. Each compound is classified into a chemical group, and volatile compounds are listed with their retention index (RI).

VID	Identity	RI	Chemical group
Pre-maceration (PM)			
0.998	TSS		
0.988	*b**		
0.982	*C**		
0.976	2-hexen-1-ol	1444	alcohol
0.975	*L**		
0.967	2-hexenal	1241	aldehyde
0.958	h^o		
0.952	acetic acid	1502	fatty acid
0.946	*a**		
0.853	octen-3-ol	1491	alcohol
-0.811	benzyl alcohol	1918	alcohol
-0.814	citronellol	1806	terpene
-0.824	*trans*-coutaric acid		hydroxycinnamic acid
-0.835	3-(methylthio)-1-propanol	1770	sulphur alcohol
-0.836	neochlorogenic acid		hydroxycinnamic acid
-0.838	ethyl decanoate	1689	ester
-0.839	ethyl 3-methylbutyl butanedioate	1931	ester
-0.840	β-phenethyl acetate	1867	ester
-0.843	malvidin 3-*O*-(6-*p*-coumaroyl)-glucoside		anthocyanin
-0.846	ethyl heptanoate	1362	ester
-0.851	isoamyl acetate	1099	ester
-0.862	phenylethyl alcohol	1946	alcohol
-0.865	malvidin 3-*O*-(6-acetyl)-glucoside		anthocyanin
-0.876	isovaleric acid	1715	fatty acid
-0.876	malvidin 3-*O*-glucoside		anthocyanin
-0.876	ethyl octanoate	1477	ester

Table 3.2. *Continuation…*

VID	Identity	RI	Chemical group
Pre-maceration (PM)			
-0.878	delphinidin 3-*O*-glucoside		anthocyanin
-0.880	1-heptanol	1496	alcohol
-0.885	ethyl acetate	859	ester
-0.891	petunidin 3-*O*-glucoside		anthocyanin
-0.893	diethyl butanedioate	1727	ester
-0.918	hexanoic acid	1876	fatty acid
-0.920	*trans*-resveratrol		stilbene
-0.923	3-methyl-1-butanol	1201	alcohol
-0.926	kaempferol glucoside		flavonol
-0.931	CI		
-0.946	caftaric acid		hydroxycinnamic acid
-0.968	astilbin		flavanonol
-0.973	syringetin-glucoside		flavonol
-0.975	ethyl 9-decenoate	1740	ester
-0.980	protocatechuic acid		hydroxybenzoic acid
-0.985	*trans*-fertaric acid		hydroxycinnamic acid
-0.986	ethyl butanoate	995	ester
-0.986	(-)-epicatechin		flavanol
-0.989	isobutanol	1054	alcohol
-0.991	syringic acid		hydroxybenzoic acid
-0.993	piceid		stilbene
-0.993	(*E*)-astringin		stilbene
-0.999	rutin		flavonol
-0.999	(+)-catechin		flavanol
-0.999	myricetin glucoside		flavonol
Maceration-alcoholic fermentation (MAF)			
0.992	*trans*-ferulic acid		hydroxycinnamic acid
0.988	isobutyl acetate	968	ester
0.987	isoquercitrin		flavonol
0.983	linalool	1594	terpene
0.982	TA		
0.982	peonidin 3-*O*-glucoside		anthocyanin
0.981	cyanidin 3-*O*-glucoside		anthocyanin
0.980	isorhamnetin glucoside		flavonol
0.972	4-methyl-1-pentanol	1335	alcohol
0.972	piceatannol		stilbene
0.964	sinapic acid		hydroxycinnamic acid
0.959	isopentyl hexanoate	1504	ester
0.933	heptane-2,3-dione	1138	ketone
0.923	isoamyl octanoate	1709	ester
0.921	malvidin 3-*O*-(6-*p*-coumaroyl)-glucoside		anthocyanin
0.912	β-phenethyl acetate	1867	ester
0.903	malvidin 3-*O*-(6-acetyl)-glucoside		anthocyanin
0.894	malvidin 3-*O*-glucoside		anthocyanin
0.892	delphinidin 3-*O*-glucoside		anthocyanin
0.890	4-hydroxybenzoic acid		hydroxybenzoic acid
0.879	petunidin 3-*O*-glucoside		anthocyanin

Table 3.2. *Continuation…*

VID	Identity	RI	Chemical group
Maceration-alcoholic fermentation (MAF)			
0.871	ethyl hexanoate	1239	ester
0.871	3-methylbutyl propanoate	1185	ester
0.865	ethyl 7-octenoate	1536	ester
0.843	ethyl decanoate	1689	ester
0.843	trans-resveratrol		stilbene
0.833	kaempferol glucoside		flavonol
0.825	CI		
0.808	ethyl dodecanoate	1880	ester
-0.897	pH		
Malolactic fermentation (MLF)			
0.998	ethyl gallate		hydroxybenzoic acid
0.993	isoamyl lactate	1621	ester
0.993	quercetin dihydrate		flavonol
0.990	caffeic acid		hydroxycinnamic acid
0.989	vanillic acid		hydroxybenzoic acid
0.989	*trans-p*-coumaric acid		hydroxycinnamic acid
0.966	gallic acid		hydroxybenzoic acid
0.959	β-damascenone	1872	norisoprenoid
0.954	ethyl 2-methylpropanoate	920	ester
0.953	*trans*-coutaric acid		hydroxycinnamic acid
0.946	neochlorogenic acid		hydroxycinnamic acid
0.928	ethyl 3-methylbutanoate	1030	ester
0.889	2,2,4-trimethyl-1,3-pentanediol diisobutyrate	1913	ester
0.889	n-decanoic acid	2211	fatty acid
0.878	diethyl butanedioate	1727	ester
0.875	1-nonanol	1706	alcohol
0.873	ethyl 2-hydroxy-4-methylpentanoate	1592	ester
0.872	3-methyl-1-pentanol	1350	alcohol
0.869	octanoic acid	2055	fatty acid
0.868	ethyl 2-methylbutanoate	1010	ester
0.847	ethyl (L)-(-)-lactate	1372	ester
0.844	ethyl 3-methylbutyl butanedioate	1931	ester
0.838	caftaric acid		hydroxycinnamic acid
0.833	2-undecanol	1762	alcohol
0.831	2-nonanol	1564	alcohol
0.830	2,6-bis(1,1-dimethylethyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	2088	ketone
0.824	α-terpineol	1753	terpene
0.807	ethyl butyl succinate	1836	ester
-0.808	chlorogenic acid		hydroxycinnamic acid
-0.837	*a**		

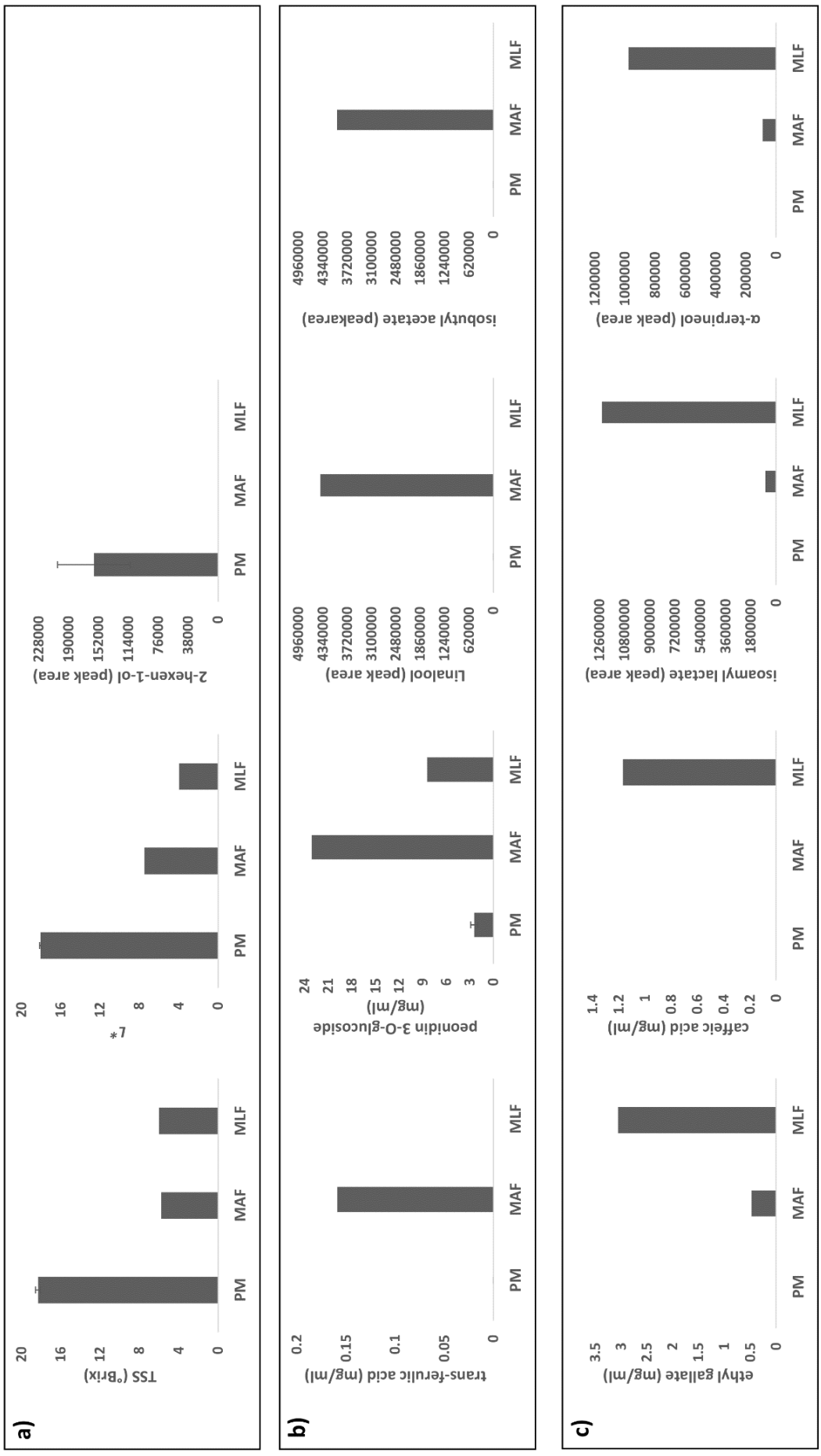

Figure 3.4 Changes in value or concentration of representative markers at each winemaking stages: pre-maceration, PM (a); maceration-alcoholic fermentation, MAF (b); and malolactic fermentation, MLF (c).

In this study, MAF samples were significantly distinguished from samples at other winemaking stages with higher amounts of anthocyanins, flavonols, stilbenes, phenolic acids (i.e. hydroxycinnamic and hydroxybenzoic acids), esters, terpene, alcohol, ketone, and also TA, and CI (**Table 3.2**). As discussed in previous sections, this phenomenon is responsible for the more intense, darker, and redder colour of MAF samples. Apart from the addition of yeast in the must to initiate the fermentation process, it is important to note that MAF process also involved a six-day continuous contact for the juice with grape skins, pulps and seeds. This allows a wide range of naturally present metabolites increasingly released from the macerated grape skins into the juice occurring as part of the mass transfer process (Pinelo, Arnous, & Meyer, 2006). The presence of alcohol due to fermentation further enhances the extraction of anthocyanins and phenolics from the vacuole of grape skins (He et al., 2012). Most of the volatiles selected after MAF have been observed and/or quantified in Merlot must, juice, and wines in several studies (Arcari et al., 2017; Hernandez‐Orte et al., 2014; Panceri et al., 2017; Song et al., 2016; Zhang et al., 2013). Terpene linalool is among the important volatile compounds providing the flowery varietal aroma to non-aromatic grape varieties such as Merlot (Maicas & Mateo, 2005), which can help differentiate Merlot wines from other red wine grape varieties (Pedroza, Zalacain, Lara, & Salinas, 2010). Linalool is one of the compounds detected exclusively in MAF samples in this study (**Figure 3.4**), which may suggest that this volatile is susceptible for extraction from the grape skins during MAF, resulting to its significant increase at this stage. However, MLF has decreased linalool, as shown in **Figure 3.4**, which can be attributed by conversion into α-terpineol (Skouroumounis & Sefton, 2000). It is also interesting to observe that 4-methyl-1-pentanol (or isohexyl alcohol) is the only alcohol compound among all the volatile markers detected in MAF samples. While a previous study has reported the detection of this volatile in Merlot wines (Zhang et al., 2013), its aroma descriptor remained unknown. On the other hand, a variety of esters (isobutyl acetate, isopentyl hexanoate, isoamyl octanoate, ethyl hexanoate, 3-methylbutyl propanoate, ethyl 7-octenoate, ethyl decanoate, ethyl dodecanoate) known to provide the fruity notes in the wine is found to be particularly dominant for MAF samples. For instance, ethyl hexanoate is linked to green apple and strawberry aromas, and ethyl dodecanoate contributes an aroma akin to candies (Arcari et al., 2017). Moreover, the detection of alcohols and esters in MAF samples can be closely link to the products generated as a result of alcoholic fermentation by yeast. Higher alcohols compounds are majorly converted from the carbon backbone of amino acids accumulated during alcoholic fermentation (Cheynier et al., 2010). Esters, on the other hand, are products of the

esterification of alcohol and acyl-CoA (derived from both amino acid and fatty acid metabolism) by the yeast's alcohol acyltransferase enzymes (Ilc et al., 2016).

With respect to MLF samples, the distinct separation of this wine sample from samples obtained at early winemaking stage is driven by a total of thirty-one discriminant markers (**Table 3.2**). Only two of these markers, chlorogenic acid and *a**, possess negative VID coefficients showing that most of the markers are detected with significantly higher amounts after MLF. The negative VID coefficient for *a** confirms the significant change in colour characteristics of the wine owing to MLF, which might be due to combined acid- and enzymatically catalysed hydrolysis and oxidation reactions (Cheynier et al., 2010). This has been a common observation in many red wine studies (Burns & Osborne, 2013; Mazza et al., 1999). Three hydroxybenzoic acids, five hydroxycinnamic acids, a flavonol, ten esters, two volatile fatty acids, a ketone, four alcohols, a terpene, and a norisoprenoid are detected in higher amounts after MLF. The origin of some of these phenolics could be those naturally present in grape while many of the compounds detected in MLF samples are generally products of the fermentation process.

The increase in hydroxybenzoic acids (e.g. gallic acid and ethyl gallate, vanillic acid) has been associated with the activities of lactic acid bacteria (LAB) during MLF (Gil-Muñoz, Gómez-Plaza, Martínez, & López-Roca, 1999). Gallic acid and ethyl gallate (**Figure 3.4**) are formed through a series of enzymatic reactions involving their precursor gallate esters. Endogenous esterases in the grape must catalyse the hydrolysis of gallate esters liberating gallic acid, and the subsequent esterification of gallic acid with ethanol would lead to production of ethyl gallate (Gil-Muñoz et al., 1999; Lingua et al., 2016). With respect to vanillic acid, the accumulation of this hydroxybenzoic acid in the wine samples might have been originated from the degradation of other forms of flavonoids (Cheynier et al., 2010).
Significant increase in the types and concentration hydroxycinnamic acids after MLF has been widely observed in previous studies (Cabrita et al., 2008; Devi, Archana, Bhavya, & Anu-Appaiah, 2017; Hernández, Estrella, Carlavilla, Martín-Álvarez, & Moreno-Arribas, 2006). For instance, a higher concentrations of caffeic acid (**Figure 3.4**) and *trans-p*-coumaric acid after MLF has also been addressed by Burns and Osborne (2013); Ginjom et al. (2011), and Hernández et al. (2006). Such increases can be explained by the cinnamoyl esterase conversion of caftaric acid in which the latter is also among one of the selected markers. An elevated activity of cinnamoyl esterase from LAB, such as *Oenococcus oeni*,

Lactobacillus plantarum, and other native bacteria in grapes, have been reported (Cabrita et al., 2008; Hernández et al., 2006). Additionally, it is possible that an increase amount of caffeic acid is attributed by the hydrolysis of chlorogenic acid by tannin acyl hydrolase, commonly referred to as tannase, leading to the major reduction of chlorogenic acid as reflected by its negative VID coefficient in **Table 3.2** (Cheynier et al., 2010; Devi et al., 2017; Martins, Roberto, Blumberg, Chen, & Macedo, 2016).

It is important to note that the majority of esters selected as markers after MLF are branched esters, such as ethyl-2-hydroxy-4-methylpentanoate, which are commonly associated with "fresh blackberry" aroma elaborated in Bordeaux wines including Merlot (Falcao, Lytra, Darriet, & Barbe, 2012). LAB has been demonstrated to synthesise branched esters, which is accompanied by the decrease in linear esters (Gammacurta et al., 2018). On the other hand, the formation of lactate esters such as ethyl lactate and isoamyl lactate (**Figure 3.4**) has been explained to associate with the lactic acid production pathway (Maicas, Gil, Pardo, & Ferrer, 1999; Zhang et al., 2013). Lactate esters contribute towards creamy notes in wine (Zhang et al., 2013).

An important norisoprenoid known as β-damascenone, contributing sweet and honey-like aroma in Merlot wines (Arcari et al., 2017; Hernandez-Orte et al., 2014) was also selected as a marker for MLF samples in this study. β-damascenone is a product of acid-catalysed hydrolysis of a number of precursors (e.g. C13-norisoprenoidic polyols) found as free volatile or as glycosides in grape skins and wine (Hernandez-Orte et al., 2009). The study of Ugliano and Moio (2006) has suggested that LAB has the ability to mediate the aforementioned hydrolysis; thus explaining the significant increase of β-damascenone in wine samples after MLF.

Two short-chain volatile fatty acids, decanoic and octanoic acids, were also selected as markers. Their increase after MLF has been reported in other red wines, such as Tempranillo (Izquierdo-Cañas, García Romero, Gómez Alonso, & Palop Herreros, 2008), Aglianico (Ugliano & Moio, 2005), Shiraz, and Pinotage (Malherbe, Tredoux, Nieuwoudt, & du Toit, 2012). It is important to note that yeast and bacteria are prone to undergo fatty acid metabolism, thus producing short-chain volatile fatty acids (Malherbe et al., 2012). When present at high concentrations, both octanoic acid and decanoic acid could lead to unpleasant

odours reminiscent of rancidity and cheese, and sourness and fatty (Arcari et al., 2017; Panceri et al., 2017; Song et al., 2016; Zhang et al., 2013).

Another two interesting markers, the alcohol 3-methyl-1-pentanol and the sulphur-containing 3-(methylthio)-propanal, are both yeast-driven volatile compounds that have been shown to increase after MLF (Malherbe et al., 2012; Ugliano & Moio, 2005). Importantly, Zhang et al. (2013) and Song et al. (2016) have identified 3-methyl-1-pentanol as potential varietal aroma for Merlot wines that contribute towards the fruity and floral aroma. On the other hand, 3-(methylthio)-propanal (methionol) is produced through yeast metabolism of the amino acid methionine, where the molecule undergoes deamination, followed by decarboxylation producing an intermediate aldehyde product 3-(methylthio)-1-propanal (methional), further reduced to the alcohol methionol (Moreira, Mendes, Guedes de Pinho, Hogg, & Vasconcelos, 2008). Interestingly, this volatile has been associated with cooked vegetables aroma in Merlot wines (Panceri et al., 2017).

Finally, an increased concentration of α-terpineol after MLF has been documented (Izquierdo-Cañas et al., 2008). As illustrated in **Figure 3.4**, a significant amount of this terpene was generated as a result of the MLF. However, despite the potential of bacterial β-glucosidase activity in synthesising α-terpineol during MLF, it was the acid-catalysed hydrolysis of geraniol considered to be more significant for generating a large amount of α-terpineol (D'Incecco et al., 2004; Skouroumounis & Sefton, 2000). Extracted from Merlot wine matrix, α-terpineol has been reported to contribute an aroma reminiscent of pine, lily, anise, and mint (Arcari et al., 2017).

3.4 Conclusion

The present work has demonstrated the potential of a multiplatform approach coupled with the state-of-the-art chemometrics to reveal those important attributes and the associated (bio)-chemical reactions being influenced the most either at pre-maceration, maceration-alcoholic fermentation or malolactic fermentation during the commercial Merlot winemaking process. It was clear that green odourant compounds (C6 compounds) were dominant prior to maceration but their concentration diminished as the fermentation progressed. Maceration-alcoholic fermentation appeared to be the most crucial vinification step affecting the phenolic and volatile composition, both qualitatively and quantitatively

since a wide range of phenolics and aroma precursors are readily extracted at this stage. A more complex and dynamic changes in the volatile and phenolic profiles of the fermenting wine was observed during malolactic fermentation. For instance, hydroxycinnamic acids and hydroxybenzoic acids were significantly increased probably due to various enzymatic and oxidation reactions while bacterial metabolism products and hydrolysis reactions led to production of new esters and alcohols volatiles. As stakeholders in the winemaking industry are constantly exploring ways to manipulate the process of transforming grapes to wine (from agronomic practices to ageing method) in order to produce quality wines, untargeted and multiplatform-based fingerprinting and profiling of grapes/wine metabolites integrated with chemometrics approach offers opportunity to fully identify how would the grape musts and their metabolites behave during the vinification process when those changes are applied.

CHAPTER 4

EFFECT OF PULSED ELECTRIC FIELDS PROCESSING ON THE EVOLUTION OF VOLATILES, PHENOLICS, AND OENOLOGICAL PROPERTIES DURING THE COMMERCIAL MERLOT WINEMAKING PROCESS

4.1 Introduction

Pulsed electric field (PEF) processing is based on the application of intermittent high voltage electric pulses for a short time (µs to ms) on a biomaterial to increase cell permeability by electropermeabilisation or electroporation, which facilitates mass transfer across cell membrane (Goldberg et al., 2018; Zimmermann, 1986). The extent of electroporation is dependent on the electric field strength and total energy applied, and the characteristics of the biological cell such as its morphology and composition (Ortega-Regules, Ros-García, Bautista-Ortín, López-Roca, & Gómez-Plaza, 2008; Raso et al., 2016a). For red grape pre-maceration treatment, electric field strength ranging from 0.7 kV/cm up to 10 kV/cm and specific energy ranging from 0.4 kJ/kg to 50 kJ/kg have been demonstrated to significantly improve extraction of colour and phenolic compounds (Oey et al., 2016; Yang, Huang, Lyu, & Wang, 2016). However these studies have been limited to laboratory scale experiments and have not yet explored variations of high electric field strengths at relatively low specific energies, advantageous for industrial scale winemaking.

It has been reported that PEF processing prior to maceration (PM) is very effective in accelerating and increasing the extraction of phenolic compounds (anthocyanins, stilbenes, and flavonoids) and aroma precursors from grapes, which are crucial in producing wines with desired colour, taste, astringency, and aroma (Delsart et al., 2014; Donsì et al., 2010; El Darra, Rajha, et al., 2016; Leong, Oey, et al., 2016; López-Alfaro et al., 2013; Teusdea et al., 2017). Garde-Cerdán et al. (2013) has also reported increased extraction of volatile compounds including monoterpenoids, esters, benzenoid compounds and little to no effect on the green odourants C_6 compounds.

Conventionally, the extraction of phenolic and aroma compounds from grape berries for red wine production occurs during simultaneous maceration and alcoholic fermentation (MAF). More metabolites are expected to leach out into the must as a function of time as the grape cells continue to disintegrate and accumulation of ethanol further enhances the solubility of various compounds (Boussetta, Vorobiev, Le, Cordin-Falcimaigne, & Lanoisellé, 2012; Leong, Burritt, et al., 2016; Setford, Jeffery, Grbin, & Muhlack, 2018; Villamor, Evans, Mattinson, & Ross, 2013). Yeast metabolism, enzymatic and chemical reactions add complexity to the fermentation process as new compounds are also being synthesised at the

same time (Cheynier et al., 2010). Nonetheless, a number of studies have utilised PEF prior to maceration and the finished red wines contained higher concentrations of anthocyanins, flavonols, tannins including smaller decondensed ones, gallic acid, (+)-catechin, (-)-epicatechin, total polyphenols, which commonly resulted to improved colour intensity, antioxidant activity, and higher probability of copigmentation and formation of derived pigments (Donsì et al., 2010; El Darra, Rajha, et al., 2016; López-Giral et al., 2015; López et al., 2008a, 2008b; López et al., 2009; Luengo et al., 2014; Puértolas, López, et al., 2010; Puértolas, Saldaña, Condón, et al., 2010; Saldaña et al., 2017). Meanwhile, the work of Comuzzo, Marconi, Zanella, and Querzè (2018) has showed that PEF can increase amounts of varietal aroma precursors from white grape berries.

Moreover, malolactic fermentation (MLF) involves the addition of lactic acid bacteria further complicates the fermenting must matrix through the conversion of malic acid into lactic acid raising the pH (Cheynier et al., 2010). MLF has been demonstrated to impact the phenolic composition and colour stability of wine (Burns & Osborne, 2013; Cejudo-Bastante, Vicario, Guillén, Hermosín-Gutiérrez, & Pérez-Coello, 2015; Martínez-Pinilla, Martínez-Lapuente, Guadalupe, & Ayestarán, 2012) and the formation, transformation, and release of volatile compounds important for wine aroma (Pérez-Martín, Izquierdo-Cañas, Seseña, García-Romero, & Palop, 2015; Ugliano & Moio, 2005, 2006). Considering the impact of MLF on the composition of the final wine, it is not known whether many of these changes are accelerated or retarded for PEF-treated grapes since information available in the literature is very limited (Puértolas, Hernández-Orte, Saldaña, Álvarez, & Raso, 2010).

Furthermore, most of the aforementioned studies using PEF as a pre-treatment focused on pre-determined attributes mostly phenolic compounds and colour, when there is a need to elucidate on the global impact of PEF on both the phenolic and volatile composition and their evolution from grapes to wine (i.e. from PM to MLF). To address these gaps, this study explores the effect of PEF treatment on Merlot grapes at each winemaking stage (i.e. PM, MAF, and MLF) and on winemaking as a whole. In order to do this, a state-of-the-art PEF equipment capable to produce high electric field strengths (33.1 – 41.5 kV/cm) at lower total energy (16.47 – 49.40 kJ/L) with commercial capacity of 500 kg/h was utilised to treat Merlot grapes prior to MAF. Investigation on the impact of PEF treatment was performed by integrating untargeted volatile fingerprinting through headspace – solid phase microextraction – gas chromatography – mass spectrometry (HS-SPME-GCMS), targeted

profiling of phenolic compounds through high performance liquid chromatography (HPLC), and measurement of colour and oenological properties. These data were combined and evaluated through multivariate data analysis (MVDA) to, ultimately, identify discriminative markers for sample differentiation.

4.2 Materials and method

4.2.1 Preparation of Merlot grape berries

Merlot grape berries were hand-picked and harvested at the winery standard maturity level (i.e. 17.80 – 19.80 °Brix; 4.92 – 5.84 g tartaric acid/L; pH 3.48 - 3.56) from Hawke's Bay region (39 °South, 176 °East; North Island, New Zealand) at the end of March 2016, and transported overnight by a refrigerated truck to a commercial winery. The grapes were stored at 5°C and destemmed using a continuous Wottle A1 grape destemmer (Anton, Poysdorf, Austria). Grape berries were slightly crushed during the destemming process and the resulting crushed grapes (or musts) were poured into an entry tank of PEF equipment.

4.2.2 PEF treatment

The grapes were treated using the continuous KEA-WEIN electroporation device (Karlsruhe Institute of Technology, Karlsruhe, Germany) connected to a 6-stage Marx generator. The electroporation reactor consists of a stainless steel parallel electrodes system with a 3.5 cm electrode gap, in a plate setup that allows a quasi-homogenous field distribution (Sack et al., 2010). The treatment volume was 57.6 cm^3 with a square cross section area of 12.5 cm^2 and the length of the treatment are was 4.7 cm. The system pressure was adjusted to approximately 0.2 MPa (2 bar) above ambient pressure in order to decrease flashover occurrence inside the electroporation reactor. The grapes were treated at a rate of 500 L/h with exponential decaying pulses for 4 different PEF processing conditions (**Table 4.1**) by adjusting the two input parameters of charging voltage per stage (25 and 33 kV) and pulse frequency (between 2 and 25 Hz) to result in different levels of output parameters for the output voltage, applied electric field strength and total required energy. Control (or No PEF treated) samples were pumped through the device without PEF treatment applied.

The temperature of the grapes was measured at both the inlet (T_{in}) and outlet (T_{out}) of the reactor. Temperature of the grape must did not exceed 24 °C during the course of PEF treatment. The pulse current applied at the reactor was measured by a Rogowski coil

connected to an oscilloscope while the electric field inside the reactor was determined by the current, the circuit parameters, and the size of area of the reactor. The total energy was calculated based on the difference in temperature at the inlet and outlet and the specific heat capacity (Sack et al., 2010), using the following *Eq. 1***.**

$$W_{spec} = c \times (T_{out} - T_{in}) \qquad (Eq.\ 1)$$

where *c* is the energy required to increase the temperature of 1 kg of grape mash by 1 K, T_{out} is the temperature at the outlet of the reactor and T_{in} is the temperature at the inlet of the reactor. In this study, *c* value was determined by heating up 1 kg of crushed grapes by 5 K while measuring the total required energy.

Table 4.1 Summary of processing parameters generated by the high-throughput PEF unit

PEF treatment ID	Input parameters		Output parameters		
	Charging voltage per stage[a] (kV)	Pulse frequency (Hz)	Output voltage[b] (kV)	Electric field strength (kV/cm)	Total energy (kJ/L)
PEF 0	0	0	0	0	0
PEF 2	25	10	116	33.1	18.90
PEF 5	25	25	116	33.1	47.25
PEF 6	33	5	145.5	41.5	16.47
PEF 8	33	15	145.5	41.5	49.40

[a] The electroporation device is equipped with a 6-stage Marx generator.
[b] Output voltage across the electrode system of the electroporation chamber is equal to the total charging voltage (i.e. charging voltage per stage multiplied with the number of stages) minus a voltage drop across the circuit inductance.

4.2.3 Merlot winemaking and sampling

All grape musts, both untreated and PEF-treated, were pumped into open top fermenters via the PEF machine. Aliquots of must samples (consisting of juice, skins and seeds) were then sampled immediately (thereafter referred as pre-maceration or "PM" sample) whereby the grape musts were strained through a kitchen grade wire mesh (1 mm^2) and the filtered juice of at least 50 mL was stored at -18 °C until further analysis. The coding for PM samples followed the PEF treatment applied to the grapes according to **Table 4.1**, i.e. PM0, PM2, PM5, PM6, PM8. These PEF treatment conditions were selected based on the previous work

by Treadwell (2016) and Leong, Treadwell, et al. (2019) that showed the effectiveness in accelerating the release of anthocyanin and phenolic compounds from the same batch of Merlot grapes.

Prior to fermentation, sulphite (50 ppm in the form of potassium metabisulphite) was added to the must followed by addition of commercial wine yeasts (*Saccharomyces cerevisiae*) to the must to initiate the alcoholic fermentation process within 24 h. The fermentation occurred between 16.5 and 25.5^{o}C in 500 kg tanks. The fermentation temperature is monitored and regulated according to the must °Brix. All sugars were completely consumed by the yeast after 7 days. More than 50 mL of grape must aliquots were sampled (thereafter referred as maceration-alcoholic fermentation or "MAF" sample) and stored at -18 °C until further analysis. The coding for MAF samples followed the PEF treatment applied to the grapes according to **Table 4.1**, i.e. MAF0, MAF2, MAF5, MAF6, MAF8.

Afterwards, the wine was pressed from the skins/seeds and malolactic bacteria was added. Malolactic fermentation took place in 50-L metal kegs. Then, the wine was racked off the lees, 30 ppm potassium metabisulphite was added and they were stored cool for 5 months at 12-13 ^{o}C. Afterwards, the wine was bottled under inert gas, sealed and stored at 4 ^{o}C until analysis (thereafter referred as malolactic fermentation or "MLF" samples). The coding for MLF samples followed the PEF treatment applied to the grapes according to **Table 4.1**, i.e. MLF0, MLF2, MLF5, MLF6, MLF8.

4.2.4 Determination of total soluble solids, pH and titratable acidity

Total soluble solids (measured as °Brix) and pH were measured using a refractometer (Atago, Tokyo, Japan) and pH meter (Hannah pH 209, Hannah Instruments, Inc., Woonsocket, USA), respectively. Titratable acidity (TA) was determined through manual titration of 5 mL juice/wine samples against 0.1 N sodium hydroxide (NaOH; AnalaR NORMAPUR®, VWR Prolabo, Leicestershire, England) with an endpoint pH of 8.2 ± 0.05. TA was calculated using *Eq. 2,* whereby N = normality; mL NaOH = volume of titrant NaOH used; mL sample = volume of sample. TA was expressed as gram of tartaric acid per litre of sample.

$$\textit{Tartaric acid (g/L)} = (\textit{N NaOH} \times \textit{mL NaOH} \times 75)/\textit{5 mL sample} \qquad \textit{(Eq. 2)}$$

Measurement of total soluble solids (TSS), pH and titratable acidity (TA) for each sample was performed in triplicates.

4.2.5 Determination of colour intensity and CIELab colour coordinates

Colour intensity (CI) and colour space coordinates *L*, a*, b*, C*,* and *h*° for each sample were measured in triplicates. CI was determined through direct measurement of the absorbance of the samples at wavelengths 420, 520, and 620 nm using UV/Vis spectrophotometer (Ultrospec 3300 Pro, Amersham Biosciences, Sweden). The sum of the absorbance was calculated as CI value (Glories, 1984). Before performing the spectrophotometric measurement, samples were centrifuged at 9335 *g* for 5 min. (IEC Micromax Centrifuge fitted with IEC 851OF Fixed-Angle Rotor, International Equipment Company, Massachusetts, USA), and the resulting supernatant was diluted appropriately. CIELAB coordinates including *L** (lightness), *a** (green to red) and *b** (blue to yellow) coordinates were measured through a HunterLab colorimeter (MiniScan EZ 4500S, Virginia, United States). Chroma (*C**, saturation) and hue (*h*°, hue) were also calculated using *equations 3 and 4.*

$$C^*_{ab} = \sqrt{a^{*2} + b^{*2}} \qquad \textit{(Eq. 3)}$$

$$h° = \arctan(b^*/a^*) \qquad \textit{(Eq. 4)}$$

4.2.6 Separation, identification and quantification of phenolic compounds using HPLC-DAD

Samples were centrifuged at 9335 *g* (IEC Micromax Centrifuge) for 5 min and the supernatant was filtered using a 0.45 µm pore size cellulose syringe filter. Chromatographic separation of phenolic compounds were carried out using a reversed-phase Kinetex C_{18} column (100 × 4.6 mm i.d., 2.6 µm pore size, 20 °C; Phenomenex, Torrance, USA) fitted with a KrudKatcher ULTRA HPLC in-line filter (0.5 µm depth × 0.10 mm internal diameter, Phenomenex) on an Agilent 1200 system (Agilent Technologies, Palo Alto, CA, USA). Mobile phase was composed of 10% (v/v) formic acid (Merck, Darmstadt, Germany) in acetonitrile (solvent A; VWR International, Pennsylvania, United States); and 10% (v/v) formic acid in deionised water (solvent B) ran in the following gradient elution conditions at a flowrate of 1 mL/min: 0-12.5 min, 96.7% B; 12.5 – 13.5 min, 64.3% B; 13.5 – 14.5 min,

96.7% B; and 14.5 – 20 min, 96.7% B. An injection volume of 5 µL sample were used and HPLC analysis was performed in six replicates for each sample. Individual phenolic compounds were identified and quantified using diode-array detector (DAD), recording UV spectra ranging from 200 to 600 nm to observe anthocyanins (520 nm), flavonols (360 nm), hydroxycinnamic acids and stilbenes (325 nm), and flavanols, a flavanonol, and hydroxybenzoic acids (280 nm). Concentration (mg/L) of individual phenolic compound (based on their peak area) was determined using external calibration curves of authentic standards (of at least 95% purity) diluted in HPLC grade methanol (Merck).

4.2.7 HS-SPME-GCMS analysis of volatile compounds

4.2.7.1 *Sample preparation, volatile extraction and GC-MS analysis*

Headspace – solid phase microextraction (HS-SPME) procedure was conducted according to Kebede *et al.* (2015) with some modifications. Juice or wine samples (2 mL) were diluted in 6 mL deionised water followed by addition of 2.5 g sodium chloride (Merck, Darmstadt, Germany) in 20 mL headspace glass vials and then capped with PTFE-coated silicon septa screw caps (Supelco, USA). Extraction of headspace volatiles was performed using DVB/CAR/PDMS-coated SPME fibre (50/30 µm, Supelco Co., Bellefonte, PA, USA) after pre-conditioning at 270 °C for 5 min. Six independent SPME extraction was performed for each sample. Equilibration of sample vials (40 °C for 5 min with agitation), extraction of volatile compounds by SPME fibre (40 °C for 30 min), and desorption (230 °C, splitless mode for 2 min, split condition for 3 min at 50:1 split ratio) were automated via the auto sampler Agilent PAL3 RSI 85 (Agilent Technologies, Inc., Santa Clara, CA, USA). Separation of volatile compounds was achieved in a ZB-Wax column (60 m × 0.32 mm inner diameter × 0.5 µm film thickness; Phenomenex) at a constant flow rate of 1 mL/min. The oven temperature was initially maintained at 50 °C for 5 min, then raised at a rate of 5 °C/min to reach 210 °C, further increased at a rate of 10 °C/min until reaching 240 °C, maintained for 5 min, and cooled down to 50 °C. Mass spectrometry of electron ionisation mode at 70 eV was used to obtain the mass spectra with a scanning range of 30-300 m/z. The MS ion source and MS quadrupole temperature were held at 230 °C and 150 °C, respectively.

4.2.7.2 *Data pre-processing for GC-MS chromatograms*

In a similar procedure used by Kebede *et al.* (2015) and Buvé *et al.* (2018), total ion chromatograms from GCMS analyses were pre-processed using Automated Mass Spectral

Deconvolution and Identification System (AMDIS; Version 2.72, 2014, National Institute of Standards and Technology, Gaithersburg, MD, USA) and Mass Profiler Professional (MPP; Version 14.9.1, 2017, Agilent Technologies, Diegen, Belgium). Peaks were identified according to NIST spectral library (NIST14, version 2.2, National Institute of Standards and technology, Gaithesburg, MD, USA) and confirmed when (a) match and reverse match is not less than 90%; (b) matching experimental and literature retention index (RI); and (c) matching retention time with standards for each chemical class.

4.2.8 Data analysis

4.2.8.1 *Univariate statistical analysis*

Statistical differences in oenological and colour properties between samples were determined through one-way analysis of variance (ANOVA), followed by Tukey's HSD (honestly significant difference) test, at $p < 0.05$ level. SPSS Statistics version 25 (IBM Corporation, New York, USA) was used to perform the univariate statistical analysis.

4.2.8.2 *Multivariate data analysis*

Data from untargeted volatile fingerprinting and targeted profiling of phenolic compounds, oenological and colour properties were integrated to produce four data sets accomplishing: (i) global differentiation of all must and wine samples according to the winemaking stage; and in-depth comparison of PEF treatment conditions (ii) at pre-maceration, (iii) after maceration-alcoholic fermentation, and (iv) after malolactic fermentation. MVDA of the attributes were conducted step-wise: (a) phenolic profile, (b) volatile fingerprint, and (c) combined phenolic profile, volatile fingerprint, colour, and oenological properties. MVDA and discriminant markers selection were performed using SOLO (Version 8.2.1, 2016, Eigenvector Research, Wenatchee, WA, USA) as discussed by Kebede et al. (2015). As a prerequisite, all data were mean-centred and weighed by their standard deviation to gain equal variance. An unsupervised exploration of the data for outliers and potential groupings was conducted through Principal Component Analysis (PCA). Central to MVDA, the correlations between individual samples and metabolites were investigated through Partial Least Squares - Discriminant Analysis (PLS-DA), where the metabolites were designated as *X*-variables and the winemaking stages (PM, MAF, and MLF) or PEF treatment (PEF0, PEF2, PEF5, PEF6, and PEF8) were assigned as the categorical *Y*-variables. Using the software OriginPro 2018 (Version 95E, Origin Lab Corporation, Northampton,

Massachusetts, USA), bi-plots were constructed adopting the minimum number of latent variables (LVs) to show class separation. Ultimately, discriminant compounds with the minimum calculated variable identification coefficient (VID) of 0.800 were selected and further linked to possible (bio)-chemical reactions.

4.3 Results and Discussion

The effect of pulsed electric fields treatment on grapes to the Merlot must and wine samples was evaluated through a multi-platform approach. Colour, oenological properties, phenolic compounds and volatile compounds (*Section 3.1*) of the samples were integrated using MVDA and were interpreted stepwise. Firstly, the data gathered were analysed over three vinification stages (PM, MAF, and MLF) to understand the influence of commercial winemaking practise on the physicochemical properties and chemical profile of musts and wines produced from PEF-treated grapes (*Section 3.2*). Secondly, the data were analysed to specify the effect of PEF treatment at varying electric field strengths and specific energies, differentiating individual samples at each vinification stage (*Sections 3.3-3.5*).

4.3.1 Oenological attributes of samples from PEF-treated and untreated grapes

Maceration and fermentation are two fundamental value-adding processes where compounds in the grape berry are extracted into the must and undergo transformations to produce wine. To check that these processes were completed, oenological properties namely, TSS, pH, and TA, were monitored. **Table 4.2** shows the changes in TSS, pH, and TA values of samples at each winemaking stage, namely pre-maceration (PM), maceration-alcoholic fermentation (MAF), and malolactic fermentation (MLF). After MAF, TSS and pH decreased, while TA increased. MLF slightly raised pH and lowered TA. Observed TSS, pH, and TA values were comparable to other studies on Merlot, PEF-treated or not (Bimpilas, Tsimogiannis, Balta-Brouma, Lymperopoulou, & Oreopoulou, 2015; Casassa, Bolcato, & Sari, 2015; González-Fernández, Marcelo, Valenciano, & Rodríguez-Pérez, 2012; Teusdea, Bandici, Kordiaka, Bandici, & Vicas, 2017). These signify the uninterrupted progression of vinification despite PEF treatments.

Table 4.2 Oenological properties and colour expressions of grape musts and wines produced

Samples	Oenological properties			
	TSS	**pH**	**TA**	**ABV**
PM0	18.33±0.27^{a}	3.47±0.01^{d}	5.14±0.03^{g}	-
PM2	18.20±0.62^{a}	3.53±0.01^{b}	5.23±0.02^{g}	-
PM5	18.53±0.99^{a}	3.55±0.01^{ab}	5.79±0.04^{f}	-
PM6	18.60±0.20^{a}	3.54±0.01^{ab}	4.94±0.02^{h}	-
PM8	18.40±0.93^{a}	3.55±0.01^{a}	5.72±0.04^{f}	-
MAF0	5.80±0.00^{bc}	3.73±0.01^{f}	9.33±0.05^{b}	-
MAF2	5.95±0.08^{b}	3.38±0.00^{f}	8.98±0.09^{c}	-
MAF5	6.00±0.00^{b}	3.42±0.01^{e}	8.92±0.06^{c}	-
MAF6	5.00±0.00^{c}	3.39±0.01^{f}	9.75±0.02^{a}	-
MAF8	6.00±0.00^{b}	3.35±0.00^{g}	8.86±0.04^{c}	-
MLF0	-	3.43±0.02^{e}	6.35±0.11^{d}	11.25±0.13
MLF2	-	3.46±0.00^{d}	6.45±0.10^{d}	11.21±0.39
MLF5	-	3.46±0.00^{d}	6.00±0.03^{e}	11.45±0.57
MLF6	-	3.45±0.00^{d}	5.96±0.02^{e}	11.47±0.13
MLF8	-	3.50±0.01^{c}	5.79±0.12^{f}	11.28±0.55

from five different PEF treatments sampled at pre-maceration (PM), after maceration-alcoholic fermentation (MAF), and after malolactic fermentation (MLF)
Data expressed as mean ± standard deviation (n=29-30). Means with the same superscript letters in the same row have no significant difference (Post Hoc Tukey test, $p < 0.05$). Oenological properties: TSS, total soluble solids (°Brix); TA, titratable acidity (g tartaric acid/L); CI, colour intensity ($A_{420} + A_{520} + A_{620}$); *L**, lightness (100, white; 0, black); *a**, redness (+, redder; -, greener); *b**, yellowness (+, yellower; -, bluer); *C**, *chroma (*+, brighter; -, duller*); h*°, hue (0° and 360°, red; 90°, yellow; 180°, green; 270°, blue).

4.3.2 **Targeted phenolic profiling (HPLC-DAD) and untargeted volatile fingerprinting (headspace-SPME-GC-MS) for grape musts and wines**

Chromatograms of representative phenolic profiles and volatile fingerprints from control and PEF-treated sample are contrasted in **Fig. 4.1** to **4.3** exposing differences in composition and peak height/area.

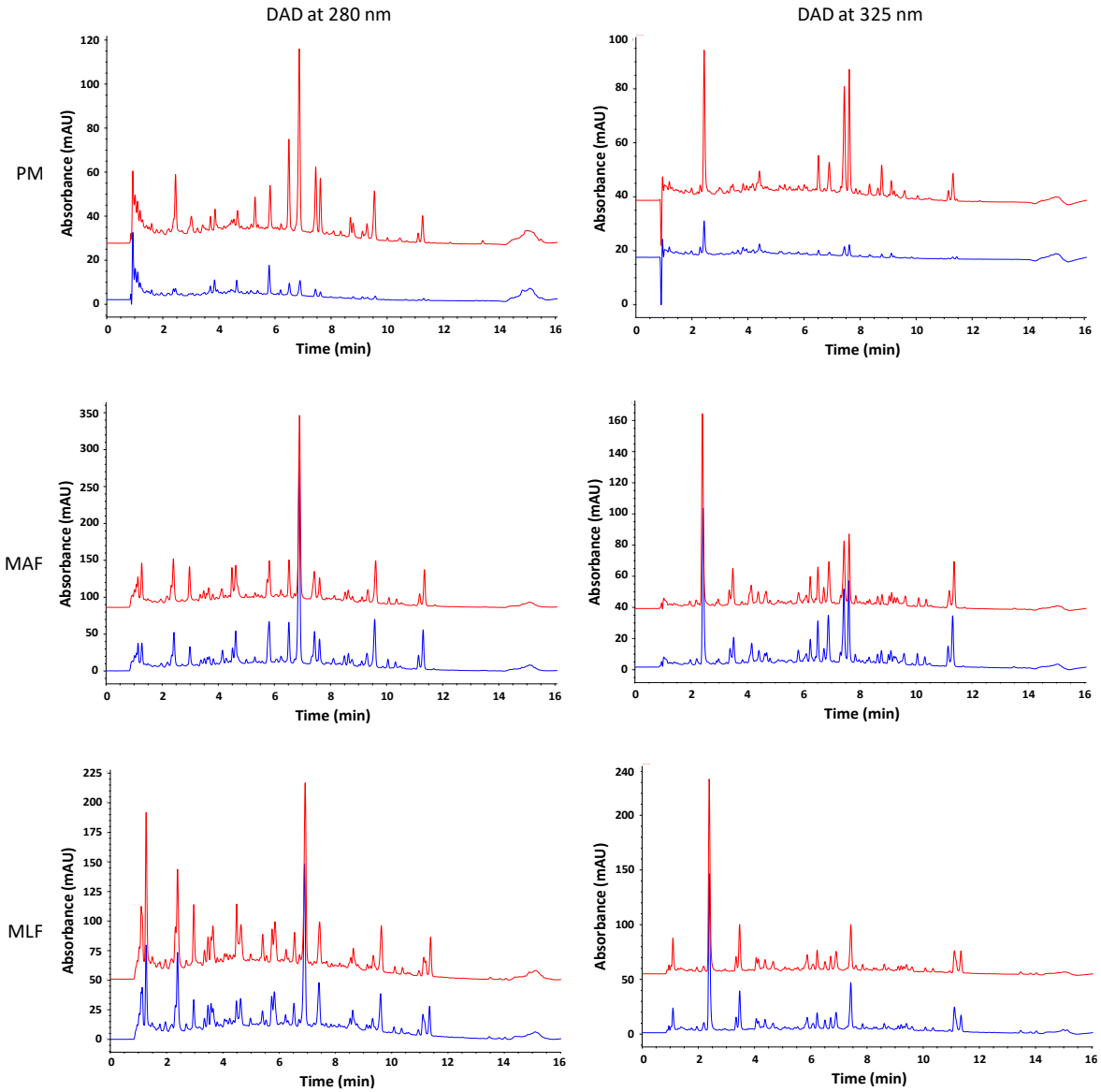

Figure 4.1 Representative HPLC-DAD chromatograms of must and wine samples from PEF-treated (PEF 8, blue line) and untreated (PEF0, red line) Merlot grapes sampled at different winemaking stages (i.e. immediately after PEF treatment (PM), after simultaneous maceration and alcoholic fermentation (MAF), and the finished wine after malolactic fermentation (MLF)). Samples were analysed at different DAD wavelengths (280 nm for hydroxybenzoic acids, flavonol, and flavononol; and 325 nm for hydroxycinnamic acids and stilbenes).

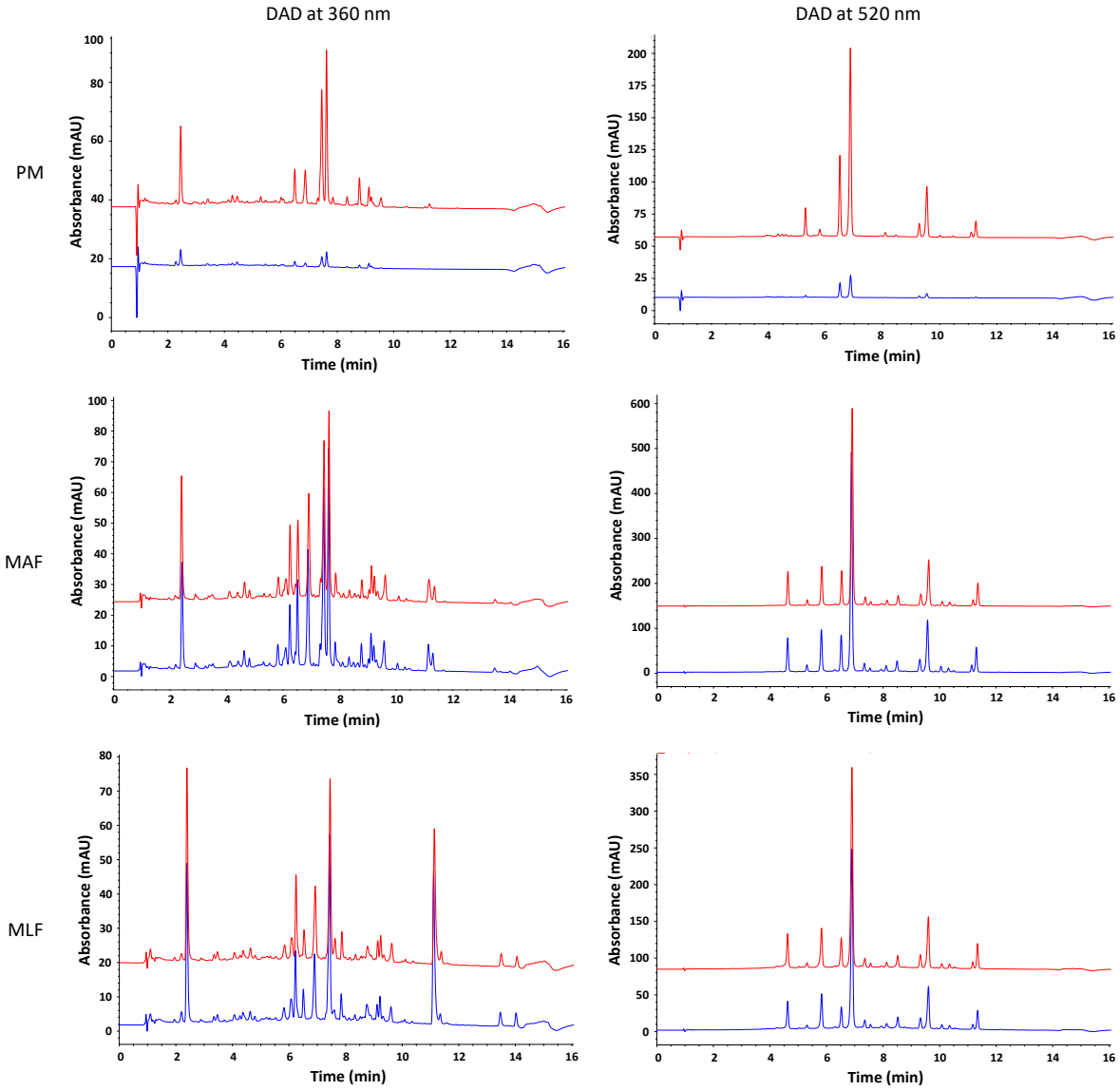

Figure 4.2 Representative HPLC-DAD chromatograms of must and wine samples from PEF-treated (PEF8, blue line) and untreated (PEF0, red line) Merlot grapes sampled at different winemaking stages (i.e. immediately after PEF treatment (PM), after simultaneous maceration and alcoholic fermentation (MAF), and the finished wine after malolactic fermentation (MLF)). Samples were analysed at different DAD wavelengths (360 nm for flavonols; and 520 nm for anthocyanins).

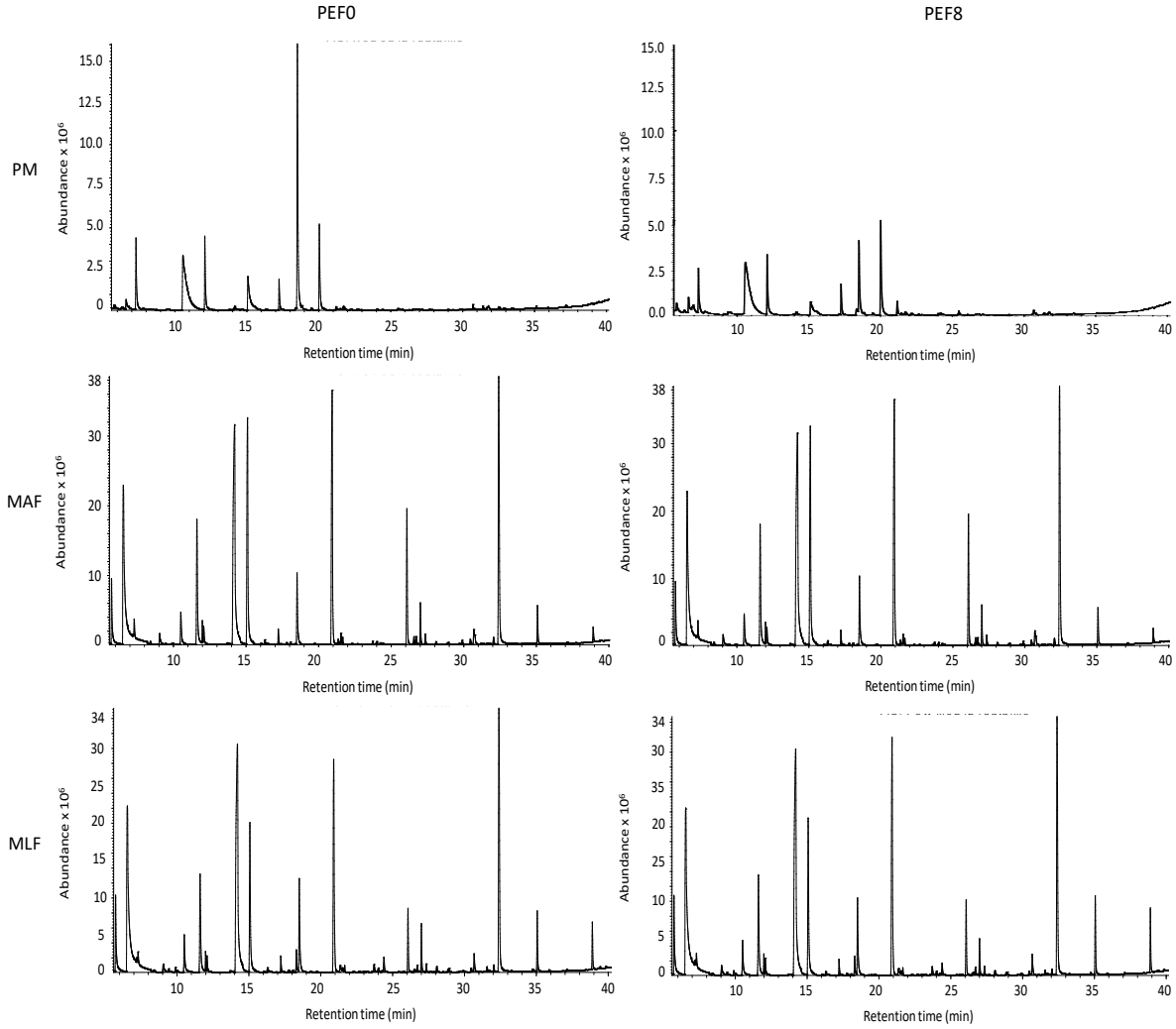

Figure 4.3 Representative total ion chromatograms (TIC) from HS-SPME-GCMS analysis of samples from PEF-treated (PEF8) and untreated (PEF0) Merlot grapes sampled at different winemaking stages (i.e. immediately after PEF treatment (PM), after simultaneous maceration and alcoholic fermentation (MAF), and the finished wine after malolactic fermentation).

4.3.3 Influence of vinification steps on the overall evolution of Merlot oenological properties, headspace volatile and phenolic profiles

To determine how each winemaking process step (i.e. pre-maceration, maceration-alcoholic fermentation, and malolactic fermentation) changes the grape must into wine and the resulting impact of PEF treatment on grapes on these changes, a comprehensive multivariate statistical data analysis (MVDA) was conducted combining more than 150 measured variables. Samples were differentiated through stepwise MVDA of (i) phenolic profiles, (ii) volatile fingerprints, and (iii) combination of all attributes determined (i.e. phenolic and volatile compounds, colour, and oenological properties).

To understand whether PEF treatment affects the transformation of Merlot juice to wine, oenological properties, headspace volatile and phenolic profiles from all 15 types of samples (5 PEF conditions and 3 winemaking stages) were modelled together into PLS-DA bi-plots. All attributes were assigned as the *X*-variables and the process steps (PM, MAF, and MLF) were designated as the categorical *Y*-variables. As illustrated in **Fig. 4.4**, the clear separation between the three winemaking process steps is driven by a large number of attributes, which seems to accumulate in the later stage of vinification (i.e. MAF and MLF). This strongly suggests uninterrupted progression of vinification of Merlot wine regardless of whether non PEF-treated or PEF-treated grapes were used as starting material.

Table 4.3 summarises attributes significantly affected for untreated and differently PEF-treated Merlot musts at each winemaking process. As reflected on the bi-plots, Merlot musts at pre-maceration are majorly characterised by the lowest concentration of phenolic and volatile compounds. Upon completion of a 7-day fermentative maceration, more phenolic compounds such as anthocyanins, flavonols, stilbenes, and a hydroxycinnamic acid were extracted and volatile compounds began to develop especially esters and higher alcohols. Through malolactic fermentation, the final wine samples had increased concentrations of hydroxycinnamic and hydroxybenzoic acids. Moreover, the volatile composition of the wine diversified with a range of fatty acid, ketone, and terpene in addition to more esters and higher alcohols. Overall, the evolution of these compounds follow three trends, where: 1) the concentration dropped dramatically after pre-maceration until the completion of MAF;

2) highest retention was achieved at MAF; and 3) gradual increase after MAF and MLF (**Figs. 4.5-4.6**).

For instance, the decreasing *L** values (**Fig. 4.5a**) indicated the darkening of samples upon transformation from juice to wine. As represented by malvidin-3-*O*-glucoside (**Fig. 4.5b**), highest concentration of anthocyanins were achieved after a 7-day fermentative maceration followed by a partial reduction, which can be attributed to deglycosylation and polymerization of monomeric anthocyanins to a more stable colour pigment in the final wine (Bartowsky, 2014; Boulton, 2001).

Similarly, substantial amount of the stilbene aglycones *trans*-resveratrol and piceatannol (**Fig. 4.5cd**) were lost after reaching maximum concentrations through MAF. Previous studies have addressed that winemaking process itself can influence the stability of stilbene aglycones. Additionally, it is also possible that isomerase enzymes from the yeast has converted *trans*-resveratrol into its *cis*-form which has not been measured in this study (Yunoki, Yasui, & Ohnishi, 2004). Despite lessened stilbene aglycones, it is important to consider the level of stilbene glycosides as several authors have reported generation of aglycones from glycoside hydrolysis (Clare et al., 2005; Vrhovsek, Wendelin, & Eder, 1997; Yunoki et al., 2004). However, results (**Fig. 4.5ef)** rather show continued increase or maintained levels of piceid and (*E*)-astringin. In finished wines, piceid can be up to three times more than *trans*-resveratrol, while (*E*)-astringin concentrations can be more than four times that of picetannol which was below detection limit. Higher glycoside levels is in agreement with previous studies, although reason is still not known (Pawlus, Waffo-Téguo, Shaver, & Mérillon, 2012; Stervbo, Vang, & Bonnesen, 2007).

On the other hand, a consistent trend has been demonstrated for most of the hydroxycinnamic and hydroxybenzoic acids where their concentration in the wine increased throughout the vinification process (**Figs. 4.5g-m**). Their significant increase during MLF may be due to the lactic acid bacteria metabolism of their precursors. Hence, the extraction of these precursors during MAF can be a determining factor for their concentration in the finished wines. For instance, the production of trans-*p*-coumaric acid during malolactic fermentation indicates the extraction of precursors (i.e. caftaric, coutaric, and chlorogenic acid) beforehand. In fact, chlorogenic acid decreased after MLF according to **Table 4.3**. Furthermore, trans-*p*-coumaric acid might also be generated form the hydrolysis of the *p*-

coumaroylated anthocyanin (e.g. malvidin-3-(6-*p*-coumaryl)-glucoside) which coincides with the decrease of the anthocyanin as exemplified in **Fig. 4.5b** (Hernández et al., 2007).

With regards to the volatile composition, no volatile compounds were common markers considered for winemaking. However, several examples were shown to show exclusivity of some compounds in a particular winemaking stage such as C6 compounds 2-hexenol (**Fig. 4.6a**) and 2-hexenal (**Fig. 4.6b**), linalool (**Fig. 4.6c**), and 2-ethyl-1-hexanol (**Fig. 4.6d**). Despite the loss of C6 compounds and linalool towards the end of winemaking, their derivatives hexyl acetate and 2-ethyl-1-hexanol (from C6 compounds, **Fig. 4.6de**) and α-terpineol (from linalool, **Fig. 4.6f**) were detected in the final wine produced (Dennis et al., 2012; Skouroumounis & Sefton, 2000).

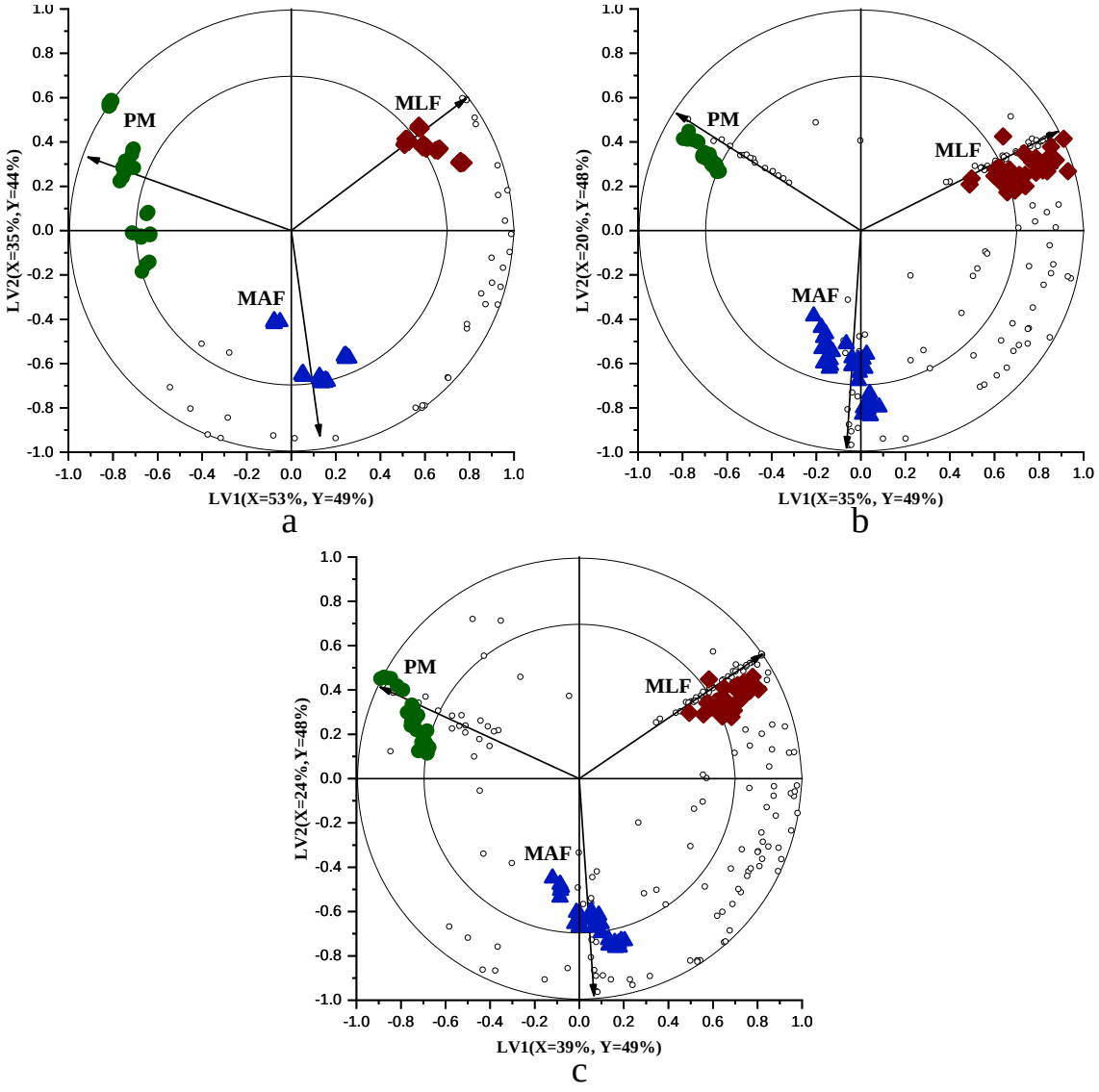

Figure 4.4 PLS-DA bi-plots illustrating the variance between PM, MAF, and MLF samples vinified either using untreated and PEF-treated Merlot grapes based on their (a) volatile profiles, (b) phenolic profiles, and (c) combined volatile profile, phenolic profiles, colour, and basic oenological properties. Classes are represented as coloured solid shapes and metabolites are drawn as open circles. Vectors signify the correlation loadings for the categorical *Y*-variables. The percentages of *X*- and *Y*-variances explained by each latent variable (LV) are specified on the respective axes.

Table 4.3 Discriminant phenolic and volatile compounds, colour, and oenological attributes selected based on VID coefficient of ≥ |0.800| for untreated and PEF-treated Merlot grapes at pre-maceration stage followed by the completion of maceration-alcoholic fermentation and malolactic fermentation. Phenolic compounds were identified and quantified with standards. The retention index (RI) for volatile compounds is also listed.

VID	Identity	RI	Chemical group
Pre-maceration (PM)			
0.973	TSS		
0.916	2-hexenol	1444	alcohol
0.838	*L**		
-0.809	ethyl decanoate	1689	ester
-0.833	ethyl 3-methylbutyl butanedioate	1931	ester
-0.835	hexanoic acid	1876	organic acid
-0.840	β-phenethyl acetate	1867	ester
-0.842	(-)-epicatechin		flavanol
-0.846	neochlorogenic acid		hydroxycinnamic acid
-0.846	isoamyl acetate	1099	ester
-0.852	3-methyl-1-butanol	1201	alcohol
-0.856	ethyl octanoate	1477	ester
-0.857	ethyl heptanoate	1362	ester
-0.858	isovaleric acid	1715	organic acid
-0.861	rutin		flavonol
-0.862	delphinidin-3-*O*-glucoside		anthocyanin
-0.866	*trans*-coutaric acid		hydroxycinnamic acid
-0.866	phenylethyl alcohol	1946	alcohol
-0.866	ethyl 9-decenoate	1740	ester
-0.867	petunidin-3-*O*-glucoside		anthocyanin
-0.869	CI		
-0.872	astilbin		flavanonol
-0.886	*trans*-fertaric acid		hydroxycinnamic acid
-0.895	ethyl butanoate	995	ester
-0.896	(*E*)-astringin		stilbene
-0.902	(+)-catechin		flavanol
-0.916	diethyl butanedioate	1727	ester
-0.929	protocatechuic acid		hydroxybenzoic acid
-0.929	caftaric acid		hydroxycinnamic acid
-0.932	ethyl acetate	859	ester
-0.947	piceid		stilbene
-0.974	syringetin glucoside		flavonol
-0.974	syringic acid		hydroxybenzoic acid
-0.976	isobutanol	1054	alcohol
-0.981	myricetin glucoside		flavonol
Maceration-alcoholic fermentation (MAF)			
0.962	linalool	1594	terpene
0.948	TA		
0.922	isoamyl octanoate	1709	ester
0.915	isorhamnetin glucoside		flavonol
0.915	isopentyl hexanoate	1504	ester
0.894	4-methyl-1-pentanol	1335	alcohol

Table 4.3 Continuation…

VID	Identity	RI	Chemical group
Maceration-alcoholic fermentation (MAF)			
0.893	ethyl 7-octenoate	1536	ester
0.890	piceatannol		stilbene
0.868	malvidin-3-*O*-(6-*p*-coumaroyl)-glucoside		anthocyanin
0.866	isobutyl octanoate	1603	ester
0.863	malvidin-3-*O*-glucoside		anthocyanin
0.863	malvidin-3-*O*-(6-acetyl)-glucoside		anthocyanin
0.861	*trans*-resveratrol		stilbene
0.848	peonidin-3-*O*-glucoside		anthocyanin
0.831	isoquercitrin		flavonol
0.822	sinapic acid		hydroxycinnamic acid
0.807	ethyl dodecanoate	1880	ester
Malolactic fermentation (MLF)			
0.988	caffeic acid		hydroxycinnamic acid
0.986	vanillic acid		hydroxybenzoic acid
0.984	*trans*-*p*-coumaric acid		hydroxycinnamic acid
0.973	quercetin		flavonols
0.953	ethyl gallate		hydroxybenzoic acid
0.952	ethyl 2-methylpropanoate	920	ester
0.947	isoamyl lactate	1621	ester
0.928	ethyl 2-methylbutanoate	1010	ester
0.925	gallic acid		hydroxybenzoic acid
0.921	*n*-decanoic acid	2211	organic acid
0.920	ethyl 2-hydroxy-4-methylpentanoate	1592	ester
0.907	*trans*-coutaric acid		hydroxycinnamic acid
0.903	2,6-bis(1,1-dimethylethyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one	2088	ketone
0.885	neochlorogenic acid		hydroxycinnamic acid
0.879	octanoic acid	2055	organic acid
0.879	ethyl (l)-(-)-lactate	1372	ester
0.876	2,2,4-trimethyl-1,3-pentanediol diisobutyrate	1913	ester
0.863	1-nonanol	1706	alcohol
0.847	2-undecanol	1762	alcohol
0.846	protocatechuic acid		hydroxybenzoic acid
0.838	3-methylbutyl 2-methylbutanoate	1311	ester
0.834	α-terpineol	1753	terpene
0.825	caftaric acid		hydroxycinnamic acid
0.825	3-(methylthio)-1-propanol	1770	alcohol
0.820	E-nerolidol	2042	terpene
0.818	1-decanol	1802	alcohol
0.814	ethyl 3-methylbutanoate	1030	ester
0.812	ethyl acetate	859	ester
0.808	3-methyl-1-pentanol	1350	alcohol
0.806	diethyl butanedioate	1727	ester
0.800	β-damascenone	1872	norisoprenoid
-0.829	chlorogenic acid	920	hydroxycinnamic acid

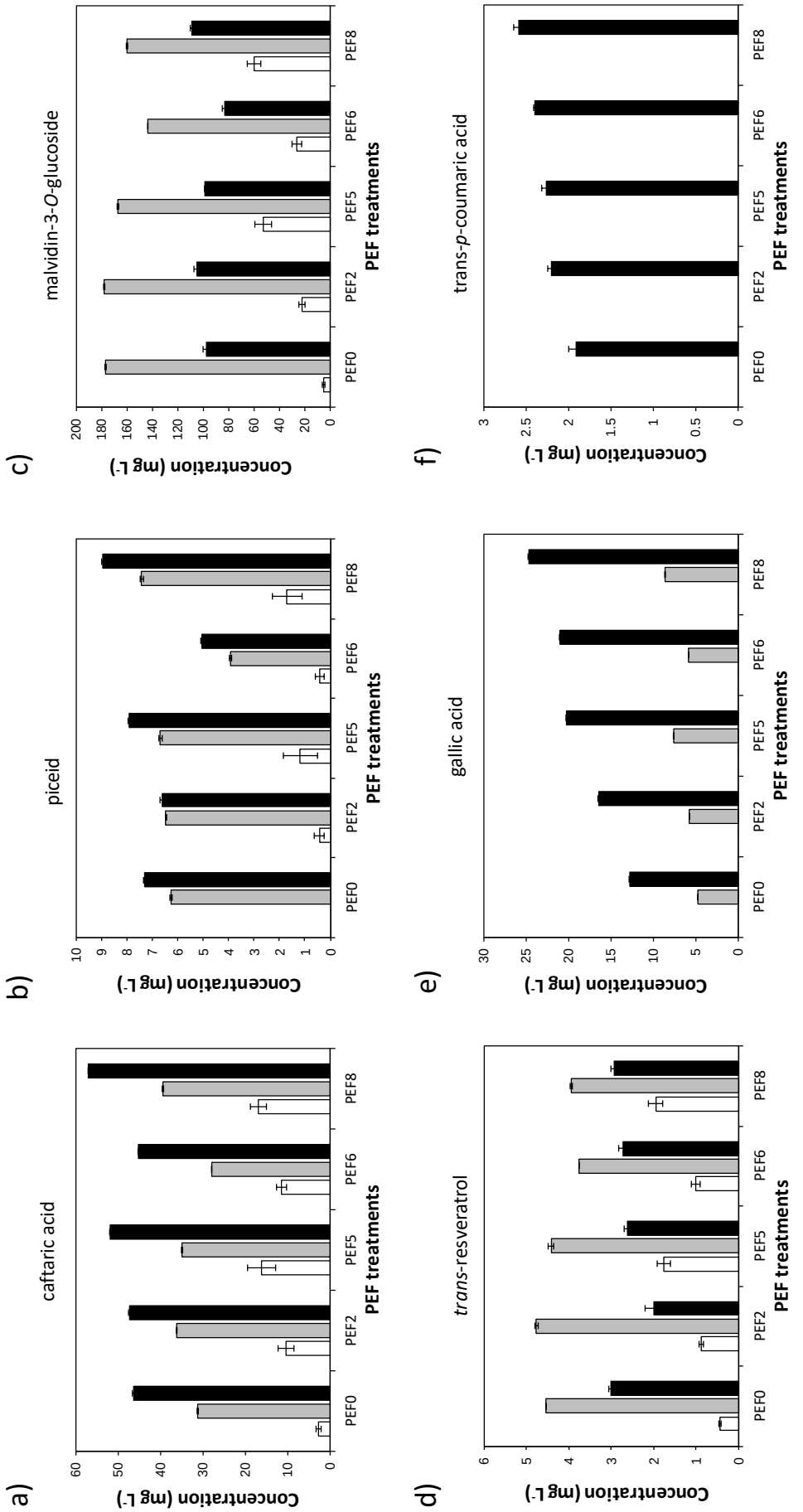

Figure 4.5 The evolution of selected phenolic compounds throughout the three stages of winemaking: pre-maceration, maceration-alcoholic fermentation, and malolactic fermentation for untreated and PEF-treated Merlot grapes. Data presented as mean ± standard deviation from six independent measurements (n = 6). Bars with different letters indicate statistically significant differences ($p < 0.05$) between the PEF treatments.

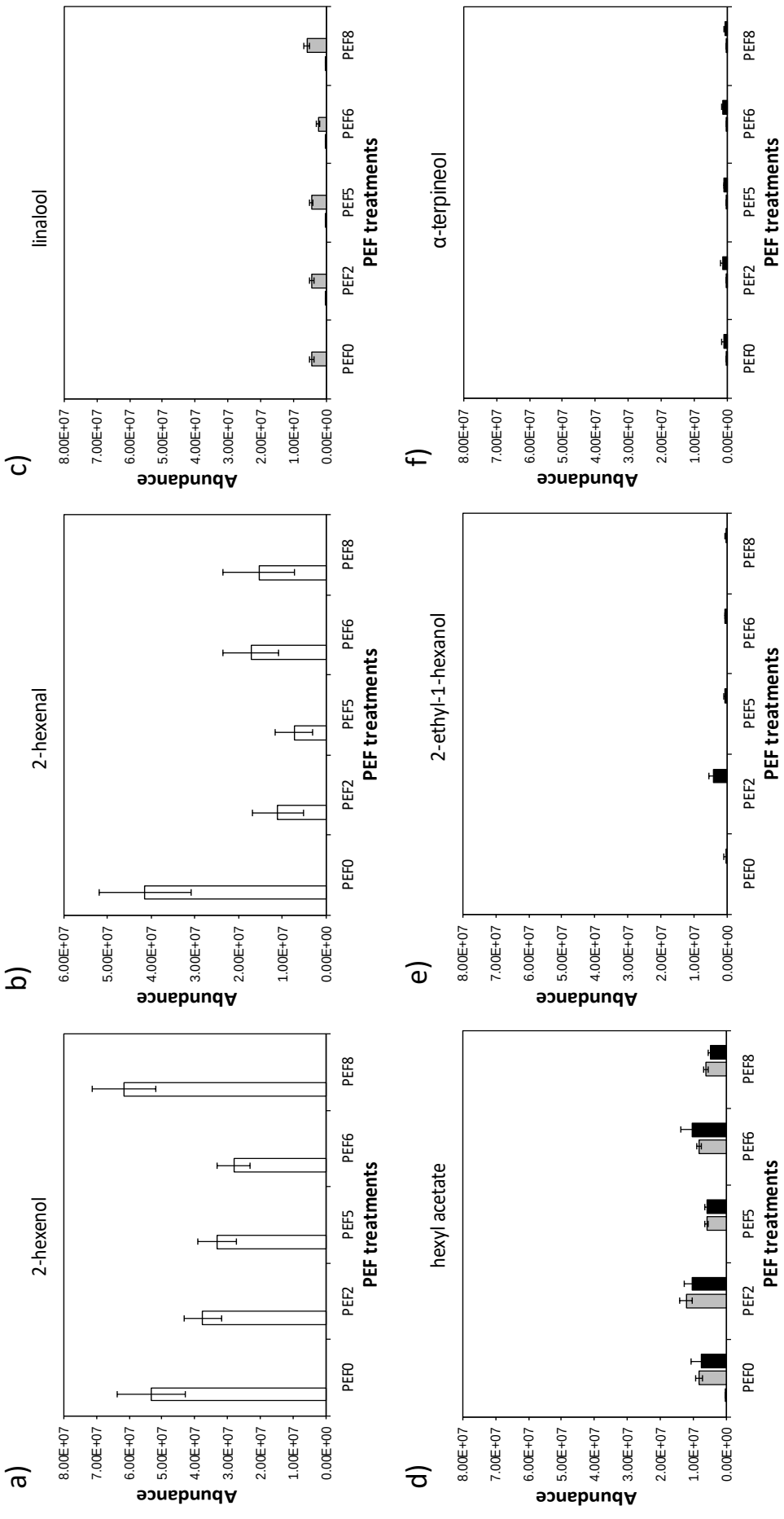

Figure 4.6 Evolution of selected volatile compounds throughout the three stages of winemaking: pre-maceration, maceration-alcoholic fermentation, and malolactic fermentation for untreated and PEF-treated Merlot grapes. Data presented as mean ± standard deviation from six independent measurements (n = 6). Bars with different letters indicate statistically significant differences ($p < 0.05$) between the PEF treatments.

4.3.4 **Pre-maceration: Immediate effect of PEF treatment on Merlot grapes**

Instantaneous PEF effect on crushed Merlot grapes could be sufficiently explained through PLS-DA models with four latent variables (LVs). **Fig. 4.7** shows the respective bi-plots of the first two latent variables. Phenolic compounds grouped the PM samples into three clusters (**Fig. 4.7a**). The first cluster was represented by the untreated grape must (PM0) samples at the far left side of the bi-plot. The second cluster, lying in the middle of the plot, includes PM2 and PM6; while the third one on the far right includes PM5 and PM8 where most phenolic compounds are projected. Interestingly, these groupings among PEF-treated samples revealed the dominating impact of specific energy over electric field strength on the extent of immediate phenolic compounds extraction. In the present work, PM5 and PM8 samples were from grapes PEF-treated at higher specific energy (47.3 - 49.4 kJ/L) compared to PM2 and PM6 (16.5 – 18.9 kJ/L). For **Fig. 4.7b**, PM0 is separated from the PEF treated samples showing that the volatile fingerprint of untreated samples is different from PEF-treated ones. However, when comparing among the PEF-treated samples, the segregation observed for volatile compounds was not as pronounced as phenolic compounds. This can be due to the low number of volatile components released or developed at this initial stage of winemaking. Usually, more aromatic headspace compounds are expected to be extracted and developed after prolonged maceration. When all analytes were considered for MVDA, as shown on **Fig. 4.7c,** the separation was visually similar to the one observed on **Fig. 4.7a**. This demonstrated that immediately after processing, the effect of PEF was dominantly influencing the phenolic compounds; in other words, the phenolic compounds drove the separation observed on **Fig. 4.7c** more than other attributes. As discussed above, this clear effect of PEF on the phenolic compounds seems to be due to the intensity of total energy applied to the musts prior to the maceration-fermentation process. This observation is in agreement with the study conducted by Leong, Burritt, et al. (2016), where release of cell contents such as phenolic compounds was demonstrated to be more prominent immediately after the grapes were subjected to a more intense energy.

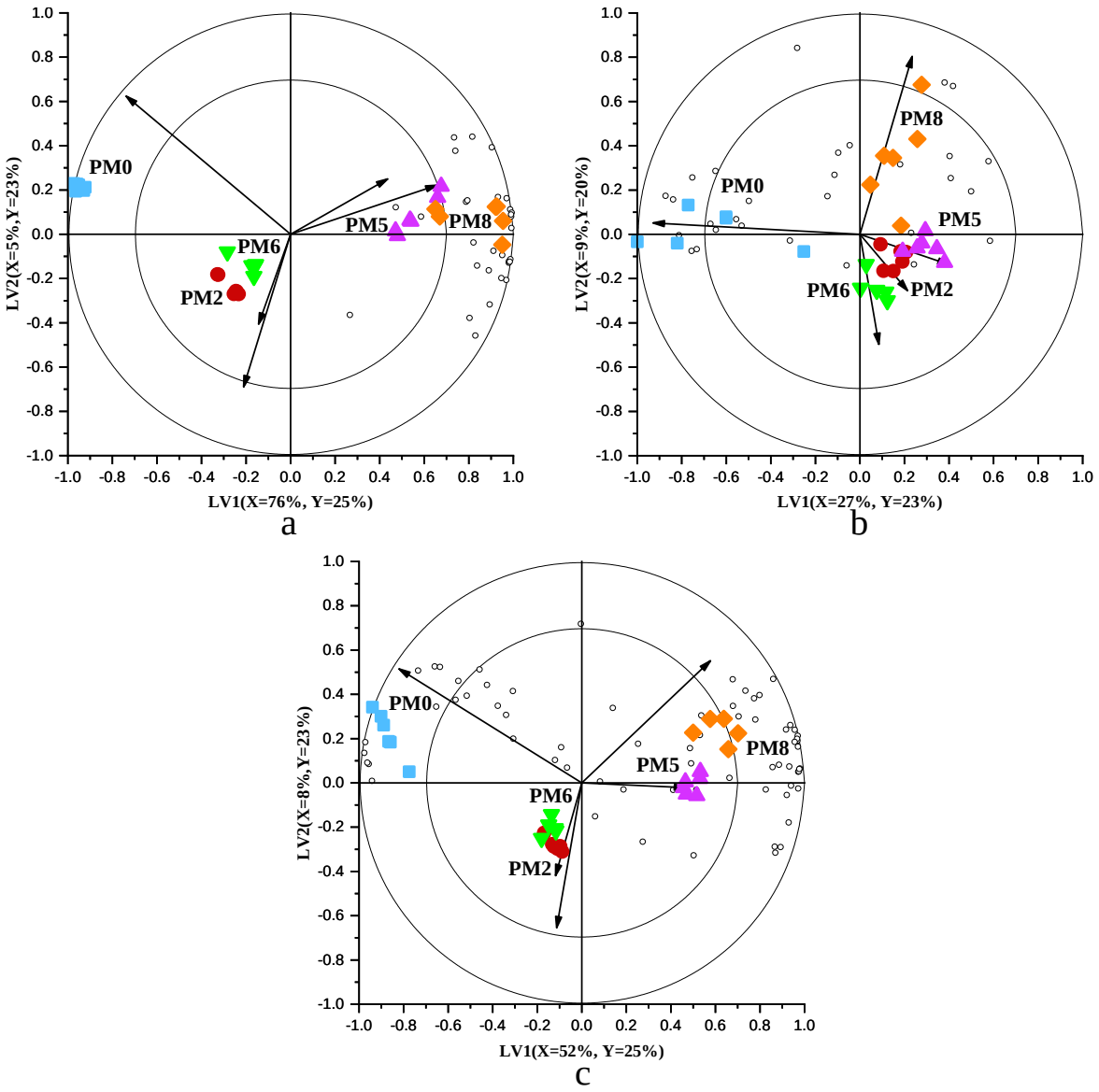

Figure 4.7 PLS-DA bi-plots comparing the immediate effect of different PEF treatments (PM2 in red, PM5 in purple, PM6 in green, and PM8 in orange) and control (PM0 in blue) on the (a) phenolic profile, (b) volatile fingerprint, and (c) combined phenolic, volatile, colour, and oenological properties of Merlot grape juice at the pre-maceration (PM) stage. Classes are represented as solid shapes and metabolites are drawn as open circles. Vectors signify the correlation loadings for the categorical *Y*-variables. The percentages of *X*- and *Y*-variances explained by each latent variable (LV) are specified on the respective axes.

To identify those analytes effectively extracted or reduced by PEF treatments, VID coefficients were calculated and discriminant markers (VID ≥ |0.800|) were selected as summarised on **Table 4.4** Analytes exhibiting positive VID coefficient signified increased concentration due to the immediate PEF treatment on the Merlot grapes, while a negative coefficient marked a decreasing trend as a result of treatment. As discussed on **Fig. 4.7c**, the separation among samples was mainly caused by the significant differences between their

phenolic compositions. In line with this observation, except for two volatile compounds (2-hexenal and linalool), all the rest discriminant markers listed in **Table 4.4** are phenolic compounds. Furthermore, the VID procedure confirmed that the high energy PEF treatments (PM8: 49.4 kJ/L and PM5: 47.3 kJ/L) appeared to immediately extract a large amount of anthocyanins, stilbenes, hydroxycinnamic acids, flavonol, flavanols, a flavanonol, and the terpene linalool from the grape berries into the juice. On the other hand, juice from untreated grapes (PM0) was found to have the lowest amount of phenolic compounds but accompanied by a high amount of green odourants such as 2-hexenal. As a consequence for high phenolic concentration, juice from grapes pre-treated with PM5 and PM8 had the darkest, most intense colour (high CI); while low amount of phenolic compounds rendered juice from PM0 grapes the lightest colour. Moreover, four major trends are observed as a result of the immediate effect of PEF treatments (**Fig.** 4.**6** and **Table 4.4)**: (1) improved extraction of compounds localised in the grape skins as represented by the increase in malvidin-3-*O*-glucoside, quercetin, and linalool; (2) darker juice colour as indicated by high CI; (3) enhanced release of seed bound compounds as represented by (+)-catechin; and (4) reduction of a green odourant, i.e. 2-hexenal.

Table 4.4 Discriminant phenolic and volatile compounds, colour, and oenological attributes selected based on VID coefficient of ≥ |0.800| for control (PM0) and four PEF treatments (PM2, 5, 6 and 8) at pre-maceration stage. Phenolic compounds were identified and quantified with standards. The retention index (RI) for volatile compounds is also listed.

VID	Identity	RI	Chemical group
PM0: Untreated			
0.981	*b**		
0.974	*h*°		
0.945	*C**		
0.944	*L**		
0.901	*a**		
0.825	2-hexenal	1241	aldehyde
-0.815	ethyl gallate		hydroxybenzoic acid
-0.830	piceatannol		stilbene
-0.846	syringetin-glucoside		flavonol
-0.847	malvidin 3-*O*-(6-*p*-coumaroyl)-glucoside		anthocyanin
-0.875	cyanidin 3-*O*-glucoside		anthocyanin
-0.888	CI		
-0.892	malvidin 3-*O*-(6-acetyl)-glucoside		anthocyanin
-0.892	*trans*-resveratrol		stilbene
-0.895	(*E*)-astringin		stilbene

Table 4.4 *Continuation…*

VID	Identity	RI	Chemical group
PM0: Untreated			
-0.896	malvidin 3-*O*-glucoside		anthocyanin
-0.907	caftaric acid		hydroxycinnamic acid
-0.908	peonidin 3-*O*-glucoside		anthocyanin
-0.916	pH		
-0.918	(+)-catechin		flavanol
-0.924	isorhamnetin glucoside		flavonol
-0.936	*trans*-coutaric acid		hydroxycinnamic acid
-0.941	rutin		flavonol
-0.943	astilbin		flavanonol
-0.943	isoquercitrin		flavonol
-0.944	sinapic acid		hydroxycinnamic acid
-0.948	neochlorogenic acid		hydroxycinnamic acid
-0.959	kaempferol glucoside		flavonol
PM2: 33.1 kV/cm, 18.9 kJ/L			
-0.828	4-hydroxybenzoic acid		hydroxybenzoic acid
PM5: 33.1 kV/cm, 47.3 kJ/L			
0.889	*trans*-resveratrol		stilbene
0.887	malvidin 3-*O*-(6-acetyl)-glucoside		anthocyanin
0.887	malvidin 3-*O*-glucoside		anthocyanin
0.865	piceatannol		stilbene
0.864	malvidin 3-*O*-(6-*p*-coumaroyl)-glucoside		anthocyanin
0.863	peonidin 3-*O*-glucoside		anthocyanin
0.856	cyanidin 3-*O*-glucoside		anthocyanin
0.847	CI		
0.843	TA		
0.838	quercetin dihydrate		flavonol
0.836	caftaric acid		hydroxycinnamic acid
0.828	sinapic acid		hydroxycinnamic acid
0.823	isoquercitrin		flavonol
0.819	astilbin		flavanonol
0.819	petunidin 3-*O*-glucoside		anthocyanin
0.816	(+)-catechin		flavanol
0.814	rutin		flavonol
0.810	delphinidin 3-*O*-glucoside		anthocyanin
0.809	kaempferol glucoside		flavonol
0.807	*trans*-ferulic acid		hydroxycinnamic acid
0.807	neochlorogenic acid		hydroxycinnamic acid
0.803	(*E*)-astringin		stilbene
-0.848	*L**		
-0.924	*b**		
-0.941	*h*°		
-0.964	*C**		
-0.976	*a**		
PM6: 41.5 kV/cm, 16.5 kJ/L			
-0.806	TA		

Table 4.4 *Continuation…*

VID	Identity	RI	Chemical group
PM8: 41.5 kV/cm, 49.4 kJ/L			
0.955	cyanidin 3-*O*-glucoside		anthocyanin
0.949	malvidin 3-*O*-(6-acetyl)-glucoside		anthocyanin
0.946	peonidin 3-*O*-glucoside		anthocyanin
0.945	astilbin		flavanonol
0.944	sinapic acid		hydroxycinnamic acid
0.942	CI		
0.939	malvidin 3-*O*-glucoside		anthocyanin
0.939	*trans*-resveratrol		stilbene
0.935	isoquercitrin		flavonol
0.934	rutin		flavonol
0.931	malvidin 3-*O*-(6-*p*-coumaroyl)-glucoside		anthocyanin
0.912	kaempferol glucoside		flavonol
0.912	quercetin dihydrate		flavonol
0.900	piceatannol		stilbene
0.897	(-)-epicatechin		flavanol
0.893	(*E*)-astringin		stilbene
0.885	isorhamnetin glucoside		flavonol
0.846	syringetin-glucoside		flavonol
0.831	linalool	1594	terpene
0.827	piceid		stilbene
0.821	caftaric acid		hydroxycinnamic acid
0.808	TA		
0.806	neochlorogenic acid		hydroxycinnamic acid
0.805	*trans*-ferulic acid		hydroxycinnamic acid
-0.800	*C**		
-0.801	*b**		
-0.814	*h*°		
-0.859	*L**		

To clearly show the major trends observed immediately after PEF treatment (at PM), individual bar plots are constructed for selected discriminant markers (see **Fig. 4.8)**. Similar to malvidin-3-*O*-glucoside, it was found that majority of phenolic compounds localised in the grape skin were released at high concentrations as a result of PEF treatments (**Fig. 4.8a**). Compared to untreated samples, treated juices, at any PEF intensity applied, were found to be at least 7 to 49 times higher in individual anthocyanins, 2-5 times in stilbenes, 2-11 times in flavonols, 4-6 times in hydroxycinnamic acids, and 4-9 times in the flavanonol astilbin. As a result of accelerated extraction of anthocyanin pigments in PEF-treated samples, the colour of the must had become darker and more intense as indicated by high colour intensity (CI) and low *L** (lightness) value in high energy PEF treatments (PM5 and PM8). High energy PEF treatments (PM5 and PM8) raised colour intensity up to 170%, while low energy

PEF treatments (PM2 and PM6) raised it by more than 50% compared to PM0 samples (**Fig. 4.8b**). Improvement in the extraction of colour pigments and various anthocyanin and phenolic compounds brought about by PEF treatment can be attributed by the reorganisation of the grape skin cell walls altering the binding sites of intracellular metabolites (e.g. polyphenols) resulting to their better release (Cholet et al., 2014). Previous studies have demonstrated that PEF treatment was generally effective in improving anthocyanins leaching (1.4 kV/cm, 20 μs pulses, 50 Hz, 20.66 ms) (Leong, Oey, & Burritt, 2016), stilbenes (7.4 kV/cm, 10-20 μs pulses, 300-400 Hz, 0.09–0.10 s) (López-Alfaro et al., 2013), flavonols and hydroxycinnamic acids (7.4 kV/cm, 20 μs pulses, 400 Hz, 0.09–0.10 s) (López-Giral et al., 2015) from a wide range of wine grape cultivars.

It was also interesting to observe that the flavonol quercetin (**Fig. 4.8c**) along with anthocyanins delphinidin-3-*O*-glucoside and petunidin-3-*O*-glucoside were detected only in juices extracted from Merlot grapes applied with high energy treatments (PM5 and PM8). This suggests that not all phenolic compounds in Merlot grapes are easily extractable and application of PEF at high energy is needed to ease their extractability. The ability of PEF in extracting the aglycon quercetin prior to alcoholic fermentation can be rather crucial to produce fine wine. Because this compound have lower polarity due to its flavonol backbones and, thus, is hardly soluble in ethanolic solution, high concentrations may result to formation of crystalline deposits in the final wine (Casassa & Harbertson, 2014). On the other hand, quercetin is useful for colour stability as it has been linked to strong copigmentation behaviour with malvidin-3-*O*-glucoside (Li, Prejanò, Toscano, & Russo, 2018), which might support the fact that juices extracted from Merlot grapes applied with high energy treatments (PM5 and PM8) having a more intense colour (**Fig. 4.8b**). In the case of the aforementioned anthocyanins, the relative low inherent concentrations (less than 10% of total anthocyanins) of delphinidin-3-*O*-glucoside and petunidin-3-*O*-glucoside in the Merlot grape skins could be the main reason contributes towards their difficulty in the extraction without any PEF pre-treatment. Nevertheless, the application of PEF particularly at high energy treatments, was evidenced to be very effective in facilitating the extraction of these low concentration anthocyanins from Merlot. In agreement with the findings from Leong, Burritt, et al. (2016), this implies the potential of PEF for extraction of specific compounds from grape skins that allow winemakers to tailor the final wine with unique characteristics compared to those wine vinified using non PEF-treated grapes.

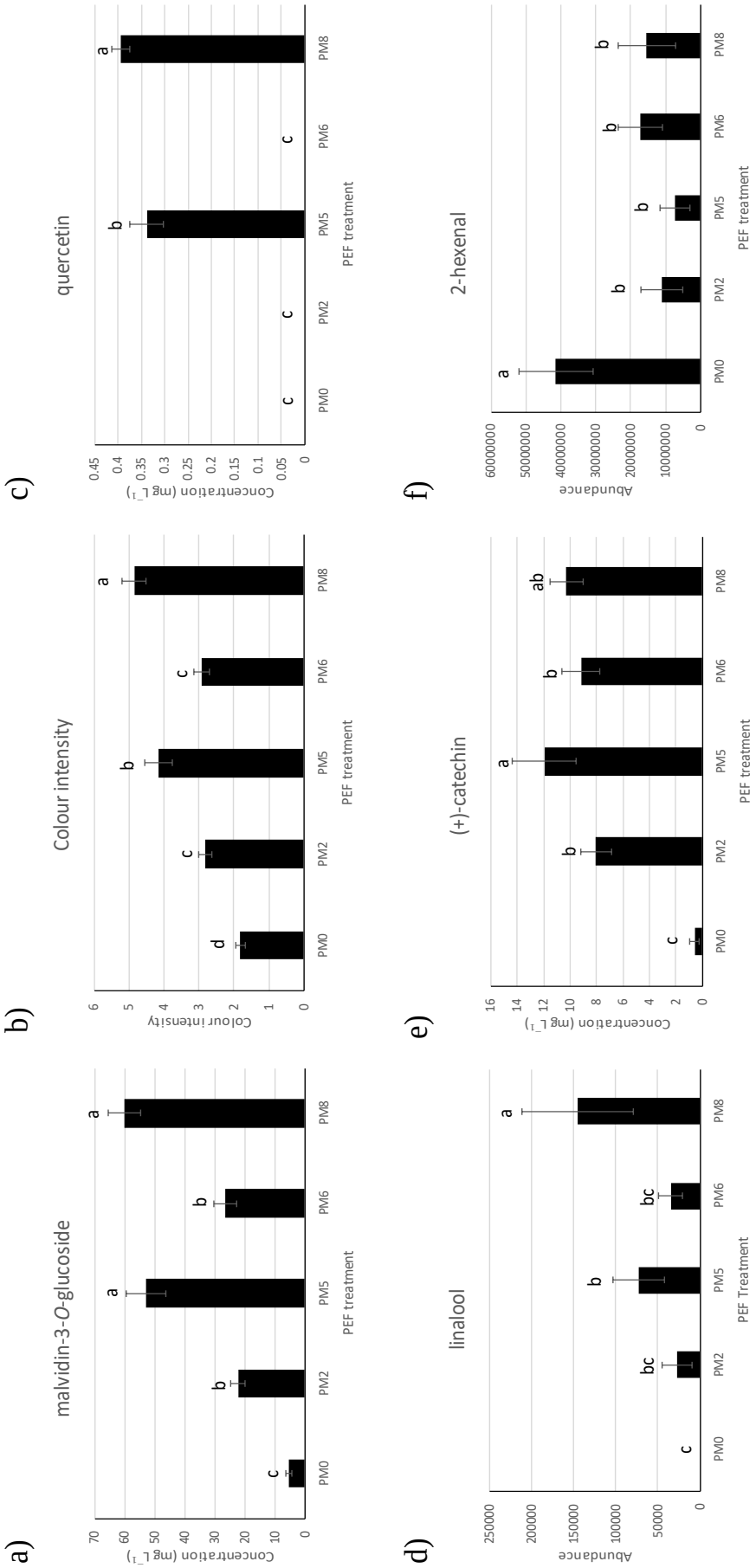

Figure 4.8 Variation in concentration or abundance value of selected discriminant markers in Merlot juice samples collected immediately after PEF treatment. Data presented as mean ± standard deviation from six independent measurements (n = 6). Bars with different letters indicate statistically significant differences ($p < 0.05$) between the PEF treatments.

In this study, it was found that terpene linalool (**Fig. 4.8d**) as well as malvidin-3-*O*-(6-*p*-coumaroyl)-glucoside, piceid, rutin, neochlorogenic acid, and ethyl gallate were only detected in the juice from PEF-treated grapes. In other words, the aforementioned compounds were absent in the juice from the untreated grapes while the application of PEF could favor their extraction. The extraction of linalool is important in the development of wine aroma because it contributes flowery varietal aroma distinguishing Merlot from other red wine varieties (Maicas & Mateo, 2005; Miguel A. Pedroza et al., 2010) and it is a precursor to α-terpineol, another volatile terpene linked to pine, lily, anise and mint aromas (Arcari et al., 2017; Skouroumounis & Sefton, 2000). While findings from this study suggested that PEF can promote the extraction linalool, the level of extraction seems to depend on the intensity of PEF applied to Merlot grapes. The application of an electric field strength of 41.5 kV/cm and total energy of 49.4 kJ/L (PM8) was the most effective in extracting the highest amount of linalool prior to maceration (**Fig. 4.8d**). Garde-Cerdán et al. (2013) have also reported the heightened extraction of linalool after PEF treatment at a field strength 7.4 kV/cm of 10 to 20 μs pulses applied at frequencies between 300 and 400 Hz on crushed Grenache red grape variety.

Apart from phenolic compounds localised in the grape skins, all PEF treatments have also improved the extraction of polyphenols majorly originating from the grape seeds such as the flavanols displayed as (+)-catechin (**Fig.** 4.**8e**). It was found that up to 21 times more (+)-catechin and three times more (-)-epicatechin was observed in the PEF-treated Merlot grapes compared to the untreated ones. Previous study by Boussetta, Lesaint, and Vorobiev (2013) has reported that the production of partial discharges in the air bubbles formed during high intensity PEF treatment at electric field strengths beyond 40 kV/cm has the capability to permeabilise the grape seed cells. Moreover, PEF treatment (7.4 kV/cm) on crushed grape berries of Tempranillo and Grenache has been shown effective in enhancing (+)-catechin and (-)-epicatechin concentrations in the must (López-Giral et al., 2015).

2-Hexenal belongs to the C_6 (six carbon) compounds linked to green, grassy aroma which is considered an off-odour in wine at high concentrations (Zalacain, Marín, Alonso, & Salinas, 2007). Most of the C_6 compounds are produced at the initial stages of winemaking via a number of enzymatic reactions involving lipoxygenase (LOX) and hydroperoxide-lyase (HPL) enzymes and the primary substrates linoleic and α-linolenic acids. These enzymatic reactions are initiatedduring grape crushing when oxygen comes in contact with the enzymes

and the substrates (Oliveira et al., 2006). In this study, a high level of 2-hexenal was found for untreated PM0 musts while a reduction in the concentration of this volatile compound was observed for all PEF-treated musts immediately after the PEF treatment (**Fig. 4.8f**). With respect to other red wine grape cultivars other than Merlot, the study of Garde-Cerdán et al. (2013) has reported a slight increase in C_6 compounds in red wines vinified using PEF-treated Tempranillo and Grenache grapes at electric field strength of 7.4 kV/cm with 10 μs pulses and frequencies between 300 and 400 Hz. Since the generation of C_6 compounds was predominantly driven by enzymatic reactions, results from the current study suggest that any PEF treatment conditions applied in this study (33.1 to 41.5 kV/cm, 16.5 to 49.4 kJ/L) are capable in reducing the activity of those endogenous enzymes, which has posed an immediate impact on the development of C_6 volatile compounds. Considering the fact that the process temperature during PEF treatment applied in this study did not exceed 25°C, it has been reported that PEF can alter the tertiary protein conformation of LOX (Luo, Zhang, Wang, Chen, & Guan, 2010).

In brief, all PEF conditions applied in this study instantaneously released significant amount of phenolic compounds and minimised the production of the green odourant 2-hexenal. Higher energy input in PEF processing, whether achieved by lower electric field strength at longer treatment time (PM5: 33.1 kV/cm, 25 Hz, 47.3 kJ/L) or by higher electric field strength at shorter treatment time (PM8: 41.5 kV/cm, 15 Hz, 49.4 kJ/L), is able to further accelerate extraction of compounds in the grape skins and seeds compared to the lower energy PEF treatments (PM2: 33.1 kV/cm, 10 Hz, 18.9 kJ/L and PM6: 41.5 kV/cm, 5 Hz, 16.5 kJ/L).

4.3.5 Maceration-alcoholic fermentation: Combined effect of PEF pre-treatment and MAF

After a 7-day simultaneous maceration and alcoholic fermentation (MAF), fermenting juice samples were collected to investigate the combined effect of PEF pre-treatment and MAF. PLS-DA bi-plots were constructed using the first 2 LVs to show the relationship between the fermented juice samples and their phenolic profiles (**Fig. 4.9a**), volatile fingerprints (**Fig. 4.9b**), and combination of all measured attributes (**Fig. 4.9c**). It was interesting to observe that the sample discrimination according to the total energy of PEF treatment as demonstrated at pre-maceration (PM) stage has shifted dramatically after seven days of fermentative maceration. After MAF, the fermented juice samples from the untreated grapes (MAF0) and pre-treated at the lowest total energy (MAF2) appeared to share similar phenolic (**Fig. 4.9a**) and volatile (**Fig. 4.9b**) compositions; thus pulling these two types of samples close together on the bi-plot (**Fig. 4.9c**). Surprisingly, MAF6 samples produced from grapes pre-treated at 41.5 kV/cm and similar range of total energy (16.5-18.9 kJ/L) as MAF 2 contained remarkably less phenolic compounds after MAF, separating it from the rest of the sample types (**Fig. 4.9a**). MAF6 was also found to develop reduced amount of volatile compounds but was sharing similar volatile fingerprint with MAF5, which is the juice sample from grapes treated at a lower electric field strength (33.1 kV/cm) than MAF6 but at a higher total energy (47.3 kJ/L) (**Fig. 4.9b**). MAF8 samples, possibly due to the high intensity PEF pre-treatment, appeared to have a more distinct phenolic and volatile composition compared to the other samples. Unlike at PM, the separation among the differently treated samples after MAF was driven by both phenolic and volatile compounds. It is important to note that the separation pattern of samples at PM was not retained after MAF (**Fig. 4.8** vs. **Fig. 4.9**), implying that the immediate effect of PEF treatment on the chemical composition of the musts was strongly influenced throughout the seven-day MAF process.

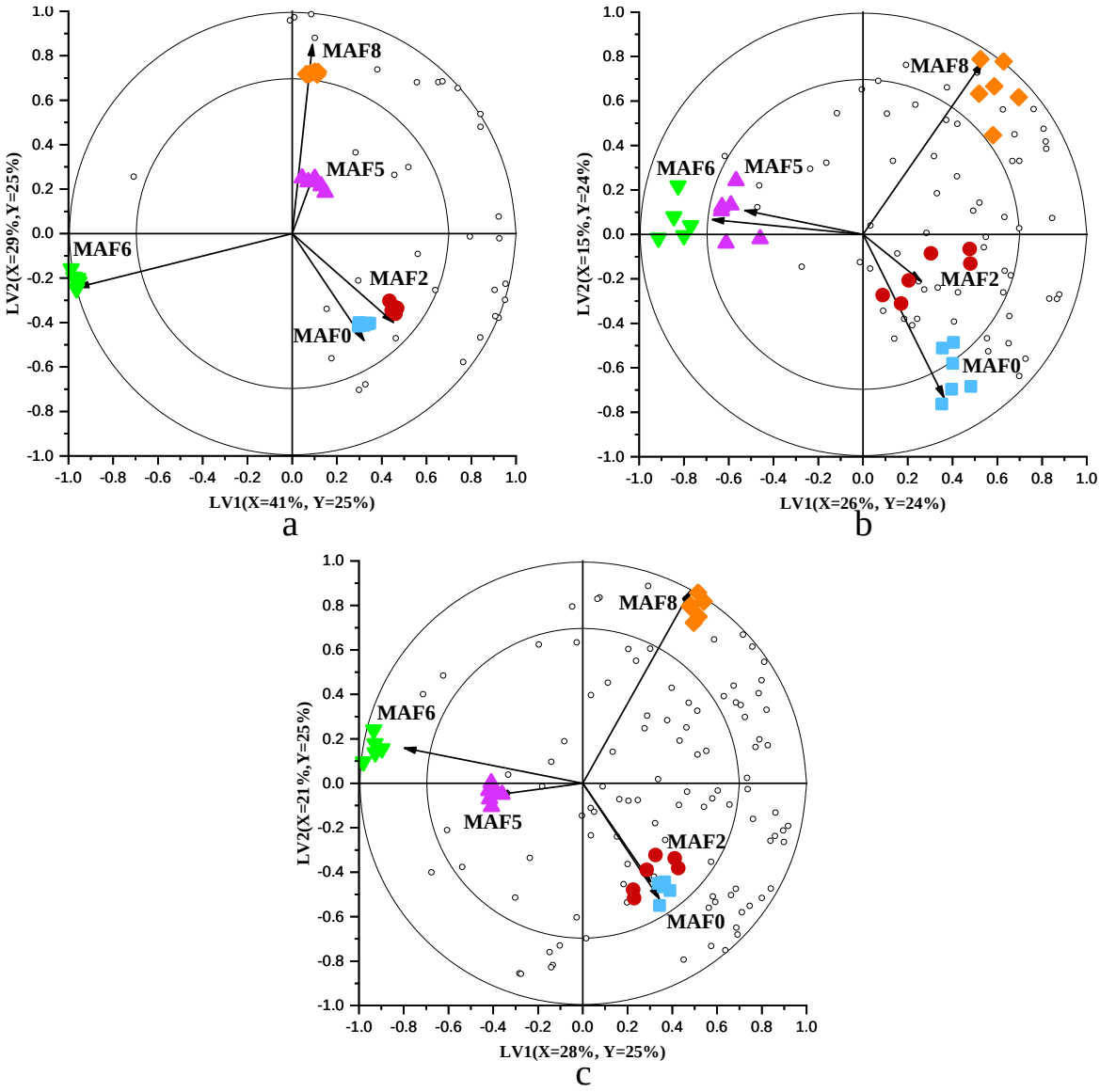

Figure 4.9 PLS-DA bi-plots comparing the combined effect of different PEF treatments (MAF0, MAF2, MAF5, MAF6, and MAF8) with MAF after six days on the (a) phenolic profile, (b) volatile profile, and (c) combined phenolic, volatile, colour, and oenological properties of fermented grape juice. Classes are represented as solid shapes and metabolites are drawn as open circles. Vectors signify the correlation loadings for the categorical *Y*-variables. The percentages of *X*- and *Y*-variances explained by each latent variable (LV) are specified on the respective axes.

Discriminant compounds (VID ≥ |0.800|) driving the separation at the end of MAF were identified and tabulated (**Table 4.5**). Based on **Table 4.5**, it was clear that high concentrations of specific species of anthocyanins and alcohols were detected in both MAF0 and MAF2 samples that drove them close together on the bi-plots. Fermented juice samples from high total energy PEF treatments (MAF5 and MAF8), regardless of the intensity of electric field strength applied, were independently characterised by high amounts of specific types of flavonols, hydroxycinnamic acid, and esters. Quite the opposite, most discriminant markers selected for MAF6 demonstrated a reduction behaviour, in which a lower concentration of anthocyanins, hydroxycinnamic acids, flavonol and stilbene was observed (**Table 4.5**). The diversity of the specific discriminant markers after MAF shows the complexity of simultaneous mass transfer and fermentation processes. Furthermore, a possible carry-over effect of the PEF pre-treatment on the raw material is likely to occur. In general, PEF-pre-treated-fermented musts have demonstrated the following behaviours: (1) modified phenolic composition of the fermenting must as exemplified by malvidin-3-*O*-(6-acetyl)-glucoside, the stilbenes trans-resveratrol, piceid, and piceatannol, and (+)-catechin (**Fig. 4.9a-e**); (2) minimised C6-compounds as exemplified by 1-hexanol (**Fig. 4.9f**); and (3) increased formation and variation of esters volatile (**Fig. 4.9g-h**).

Table 4.5 Discriminant phenolic and volatile, colour, and oenological attributes selected based on VID coefficient of ≥ |0.800| for fermenting juice samples from untreated (MAF0) and PEF-treated (MAF2, 5, 6 and 8) grapes. Phenolic compounds were identified and quantified with standards. The retention index (RI) for volatile compounds is also listed.

VID	Identity	RI	Chemical group
MAF0: Untreated			
0.951	CI		
0.858	kaempferol glucoside		flavonol
0.833	1-hexanol	1380	higher alcohol
0.831	cyaniding-3-*O*-glucoside		anthocyanin
-0.867	ethyl gallate		hydroxybenzoic acid
MAF2: 33.1 kV/cm, 18.9 kJ/L			
0.857	1-heptanol	1496	higher alcohol
0.825	petunidin-3-*O*-glucoside		anthocyanin
0.823	malvidin-3-*O*-(6-p-coumaroyl)-glucoside		anthocyanin
0.816	hexyl acetate	1290	ester
0.803	malvidin-3-*O*-(6-acetyl)-glucoside		anthocyanin
0.800	trans-resveratrol		stilbene
MAF5: 33.1 kV/cm, 47.3 kJ/L			
0.917	quercetin dihydrate		flavonol
0.856	3-methylbutyl pentadecanoate	1898	ester
0.853	pH		
0.808	piceatannol		stilbene
0.801	chlorogenic acid		hydroxycinnamic acid
-0.961	protocatechuic acid		hydroxybenzoic acid
MAF6: 41.5 kV/cm, 16.5 kJ/L			
0.818	TA		
-0.800	peonidin-3-*O*-glucoside		anthocyanin
-0.817	malvidin-3-*O*-(6-p-coumaroyl)-glucoside		anthocyanin
-0.830	trans-coutaric acid		hydroxycinnamic acid
-0.845	malvidin-3-*O*-glucoside		anthocyanin
-0.864	petunidin-3-*O*-glucoside		anthocyanin
-0.870	neochlorogenic acid		hydroxycinnamic acid
-0.885	delphinidin-3-*O*-glucoside		anthocyanin
-0.895	myricetin glucoside		flavonol
-0.924	piceid		stilbene
-0.952	TSS		
MAF8: 41.5 kV/cm, 49.4 kJ/L			
0.952	trans-fertaric acid		hydroxycinnamic acid
0.923	diethyl butanedioate	1727	ester
0.898	(+)-catechin		flavanol
0.895	syringetin-glucoside		flavonol
0.864	isoamyl isobutanoate	1189	ester
0.813	ethyl butanoate	995	ester
-0.882	*C**		
-0.884	*a**		

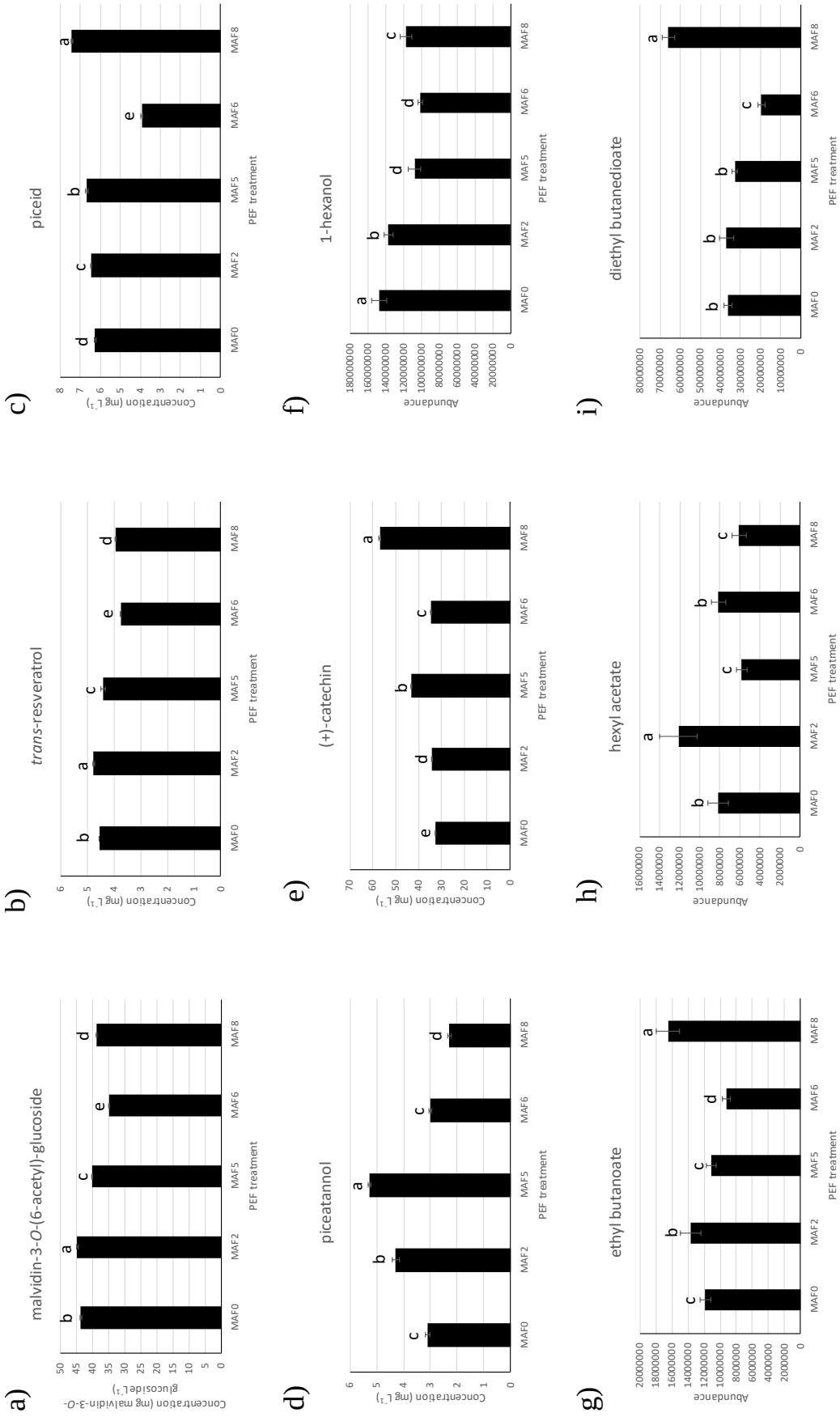

Figure 4.10 Variation in concentration or abundance value of selected discriminant markers in fermenting juice samples collected after simultaneous maceration and alcoholic fermentation (MAF) of musts from untreated and PEF-treated Merlot grapes. Data presented as mean ± standard deviation from six independent measurements (n = 6). Bars with different letters indicate statistically significant differences ($p < 0.05$) between the PEF treatments.

Malvidin glycosides are the most abundant anthocyanins in *Vitis vinifera* grapes, and accounts for more than 50% of anthocyanins in Merlot grape skins (Dimitrovska, Bocevska, Dimitrovski, & Murkovic, 2011). Findings from this study showed that the concentrations of malvidin-3-*O*-(6-acetyl)-glucoside (**Fig. 4.10a**) alongside with anthocyanins malvidin-3-*O*-glucoside and petunidin-3-*O*-glucoside were the highest for juices pre-treated at low PEF energy (MAF2). However, juices from untreated (MAF0) and other PEF-treated grapes at a higher total energy (MAF5 and 8) intensity or higher electric field strength (MAF6) exhibited lower anthocyanin concentrations. The reason that each type of juices exhibited varying level of anthocyanins even though the corresponding grapes experienced similar duration of MAF could be because PEF pre-treatments applied on Merlot grapes in this study have influenced the extractability rate of anthocyanins from the skin cells. Previous study from Leong, Burritt, et al. (2016) has evidenced that PEF-treated grapes usually released anthocyanins faster, thus achieved the adsorption-desorption equilibrium between the anthocyanins inside the skin and outside the juice within a shorter duration (50% time reduction). Therefore, it is reasonable to assume that the maximum level of anthocyanin extractability might have been pushed earlier than seven days for PEF-treated grapes, particularly for those treated at high-energy PEF treatments. Consequently, it is likely that after anthocyanin achieved the highest level in fermenting juice samples from PEF-treated grapes, the monomeric anthocyanins start to decline due to formation of derived pigments through condensation and copigmentation, oxidation, and adsorption on yeast cells (Aleixandre-Tudo & du Toit, 2018; Setford, Jeffery, Grbin, & Muhlack, 2017). It is clear from this finding that extending the MAF process up to 7 days for PEF-treated Merlot grapes, particularly for those treated at high intensity, does not render to a high amount of anthocyanins retention as the optimum extractability of anthocyanins might have been achieved during the early stage of MAF. Therefore, reducing the length of fermentative maceration of red grape varieties after a PEF pre-treatment would be preferable.

In the present study, four types of stilbenoids were detected in the Merlot juice namely trans-resveratrol, its glycoside form piceid, piceatannol and its glycoside form astringin. Stilbenes are currently recognised for their biological activities such as antioxidant, antimicrobial, anti-inflammatory, anti-ageing, vasoprotective, anticancer, antidiabetic, and neuroprotective potentials (Flamini & De Rosso, 2018; López-Alfaro et al., 2013). With the exception of astringin, most stilbenoids were identified as important markers revealing a strong difference between the fermented juices from differently PEF-treated grapes after a 7-day MAF process

in this study (**Table 4.5**). Moreover, **Figs. 4.10b-d** clearly show that not all stilbenoids behaved similarly after each PEF treatment, leading to variation in their retention in the fermenting juice after the MAF process. A higher concentration of *trans*-resveratrol (**Fig. 4.10b**) was found in the samples from untreated and low intensity PEF-treated grapes (MAF0 and MAF2) compared to the samples from high intensity PEF-treated grapes (MAF5 and MAF8) with the reversed trend observed for piceid retention in the respective fermented juices (**Fig. 4.10c**). This could be explained by two reasons. Firstly, the accelerated extraction of *trans*-resveratrol immediately after PEF treatment (see PM markers in **Table 4.4**) combined with its instability might have exposed resveratrol to oxidation, transformation to other new compounds, yeast adsorption, and yeast metabolism long before MAF finishes (Clare, Skurray, & Shalliker, 2005; Guerrero, Puertas, Jiménez, Cacho, & Cantos-Villar, 2010). The work of Sun, Ribes, Leandro, Belchior, and Spranger (2006) has also demonstrated that the level of resveratrol in the grape skin does not correlate to the amount found in the corresponding wine due to its instability, while the opposite is applicable to piceid, which is a more stable glycosylated form of resveratrol and, hence, more readily extractable from the grape skin. Coinciding with the findings on anthocyanins, optimum retention of resveratrol in the fermenting juice can be achieved by optimising the length of fermentative maceration when PEF is applied on the grapes. Secondly, the presence of endogenous β-glucosidase in grapes responsible for hydrolysing piceid into resveratrol (Mattivi, Reniero, & Korhammer, 1995), might have been affected by the initial high-energy PEF treatments. The work of Aguiló-Aguayo, Sobrino-López, Soliva-Fortuny, and Martín-Belloso (2008) has reported reduced activity of this enzyme at high intensity PEF treatments (35 kV/cm, 5512 kJ/L) on strawberry juice. Nevertheless, despite a reduced β-glucosidase activity, high amount of piceid can still influence resveratrol concentration as acid hydrolysis can gradually liberate the aglycone further into winemaking and ageing (Mattivi et al., 1995). On the other hand, the level of piceatannol in the fermented juice seemed to be more protected from degradation during the 7-day MAF for grapes that were PEF-treated at electric field strength of 33.1 kV/cm (MAF2 and MAF5) compared to those treated at 41.5 kV/cm (MAF6 and MAF8), regardless of the total energy applied (**Fig. 4.10d**). Overall, fermented juice samples produced from PEF-treated grapes at 33.1 kV/cm and 47.3 kJ/L (MAF5) appeared to maintain the highest total amount of stilbenes quantified (sum of resveratrol, piceid, piceatannol, and astringin) after 7 days MAF.

The ability of PEF in extracting a large amount of (+)-catechin prior to MAF process has been demonstrated earlier at PM (see **Fig. 4.8e**). Even after the PEF-treated grape musts undergo a 7-day long MAF process, a high level of (+)-catechin was continuously extracted from the grape musts pre-treated with high-energy PEF treatments (47.3 – 49.4 kJ/L), with MAF5 and MAF8 samples extracting at least 32% and 75% respectively more than the untreated samples (**Fig. 4.10e**). This clearly emphasised a strong carry-over effect of PEF from the early stage of winemaking to the completion of MAF process. It is also important to note that the applied high-energy PEF treatments in this study were very effective on affecting the cell permeability of grape seed as (+)-catechin is generally accumulated in the seed and not at the grape skin. The reason that previous studies were unable to achieve high level of (+)-catechin concentration in their final wine is probably due to the application of low electric field strengths on grapes (5 – 7.5 kV/cm) (El Darra, Turk, et al., 2016; López-Giral et al., 2015; Luengo et al., 2014) compared to the intensity applied in this study (33.1 and 41.5 kV/cm). (+)-Catechin is among the flavanols responsible for the taste, astringency, and colour as a cofactor in red wines (Boulton, 2001; Cassino, Tsolakis, Bonello, Gianotti, & Osella, 2019).

At PM, PEF treatment has been suggested to partially deactivate LOX and HPL resulting to reduction of a C_6 compound, i.e. 2-hexenal (see **Fig. 4.8f**). In agreement with the earlier finding, a higher level of 1-hexanol, a C_6 higher alcohol contributing to herbaceous aroma, was found in untreated samples compared to all other PEF-treated samples (**Fig. 4.10f**). This clearly signify sustained higher activities of LOX and HPL during the MAF process in the non PEF-treated sample compared to PEF-treated samples. To the best of our knowledge, this is the first time PEF treatment, albeit application at higher electric field strength for a red grape variety, has been demonstrated to minimise certain C_6 compounds from pre-maceration until after fermentative maceration. Another reason to consider could be the possible suppressing effect on the volatility of C_6 compounds due to the modified phenolic composition of the samples from PEF-treated grapes. Several authors (Lund, Nicolau, Gardner, & Kilmartin, 2009; Robinson et al., 2009; Rodríguez-Bencomo et al., 2011; Villamor et al., 2013) have substantiated the influence of the wine matrix on the volatility of aroma compounds. Therefore, further investigations comparing concentrations against odour threshold and/or sensory evaluation should be conducted to ascertain the potential impact of the extent of C_6 compounds reduction by PEF on the wine quality.

The combined effect of PEF pre-treatment on the grapes followed by a 7-day MAF process has also increased abundance and variation of esters in the resulting fermenting juices (**Table 4.5, Figs. 4.10g-i**). For MAF2, the detection of 1-heptanol has been linked to fruity, mouldy, and musty odour (Song et al., 2016; Zhang et al., 2013), while hexyl acetate has been associated with fruity, herbs, apple, pear, and cherry smells (Arcari et al., 2017; Varela et al., 2017). The ester 3-methylbutyl pentadecanoate characterised in MAF5 sample has been strongly linked to the action of enzymes from the yeast (e.g. xylanase, amylase, and pectinase) (Maturano et al., 2015). For MAF8, only ethyl butanoate out of the three esters was detected in Merlot grapes before, which contributes fruity and strawberry aroma (Arcari et al., 2017; Varela et al., 2017). The variation of esters and higher alcohols found in the wine can be usually traced back to the differences in the extracted primary metabolites or precursors, particularly amino acids and fatty acids from the grape skins, pulps or seeds (Robinson et al., 2014). For instance, the presence of additional amount of amino acids in Merlot musts has been demonstrated to increase higher alcohols, and esters during alcoholic fermentation (Hernández-Orte, Ibarz, Cacho, & Ferreira, 2006). This is because free amino acids make up the majority of nitrogen containing compounds that will directly influence the yeast assimilable nitrogen requirements of a grape must important for successful alcoholic fermentation. With respect to red grape variety, the inclusion of red grape skins during the maceration-fermentation process further increases amino acids and fatty acids, as red grape skins are rich source of amino acids (Lee & Schreiner, 2010). Therefore, it is reasonable to assume that with the same starting grape material and yeast strain utilised in this study, PEF pre-treatments applied at varying intensities are likely to modify the composition of primary metabolites or precursors extracted from the Merlot berries into the must that led to the development of a wide variety of esters during the fermentation process. In agreement, study by Garde-Cerdán, Arias-Gil, Marsellés-Fontanet, Ancín-Azpilicueta, and Martín-Belloso (2007) found increased concentrations of amino acids and fatty acids in juice from PEF-treated (35 kV/cm) Parellada white grapes.

In general, PEF treatment has modified the extractability of phenolic compounds and aroma precursors influencing the interaction between compounds, enzymes, and yeasts and the resulting composition of the fermenting must during the 7 days MAF process.

4.3.6 Malolactic fermentation: Influence of PEF as pre-treatment on the composition of the finished wine

As can be seen from the PLS-DA bi-plots (**Fig. 4.11)**, the separation between the wine samples was dominated by the differences in phenolic profiles rather than the headspace volatiles. Phenolic compounds grouped the MLF samples into three clusters: (i) MLF0; (ii) MLF2 and MLF6; and (iii) MLF5 and MLF8. Interestingly, a similar groupings was observed on PM samples immediately after PEF processing (**Fig. 4.7** vs. **Fig. 4.11**). This clearly shows a strong carry-over effect of PEF from the early stage of winemaking to the completion of MLF process, especially on the phenolic compounds.

In agreement with the bi-plots, most of the discriminant markers listed in **Table 4.6** are phenolic compounds. The markers selected through the VID procedure for MLF0 wine suggested that the final wine produced using untreated Merlot grapes was characterised by a high level of neochlorogenic acid and a lower amount of gallic acid and *trans*-*p*-coumaric acid (**Table 4.6**). However, an opposite trend for these phenolic acids was found in final wines vinified using differently PEF-treated Merlot grapes (**Figs. 4.12a-b**). The reduced amount of neochlorogenic acid in wines vinified using PEF-treated grapes might result from the copigmentation phenomena with the extracted anthocyanins (Boulton, 2001). On the other hand, a higher amount of gallic acid and trans-*p*-coumaric acid in the MAF2, 5, 6 and 8 wines suggested that PEF may have improved the extraction of their corresponding precursors. For example, gallate esters from flavanols can form gallic acid (Gil-Muñoz et al., 1999; Lingua et al., 2016) while caftaric, coutaric, and chlorogenic acid can be transformed into trans-*p*-coumaric acid (Cabrita et al., 2008; Devi et al., 2017; T. Hernández et al., 2007; Lekha & Lonsane, 1997; Martins et al., 2016). It was likely that a PEF pre-treatment on Merlot grapes favoured such phenolic transformations in the musts under the alcoholic-maceration and malolactic fermentation conditions.

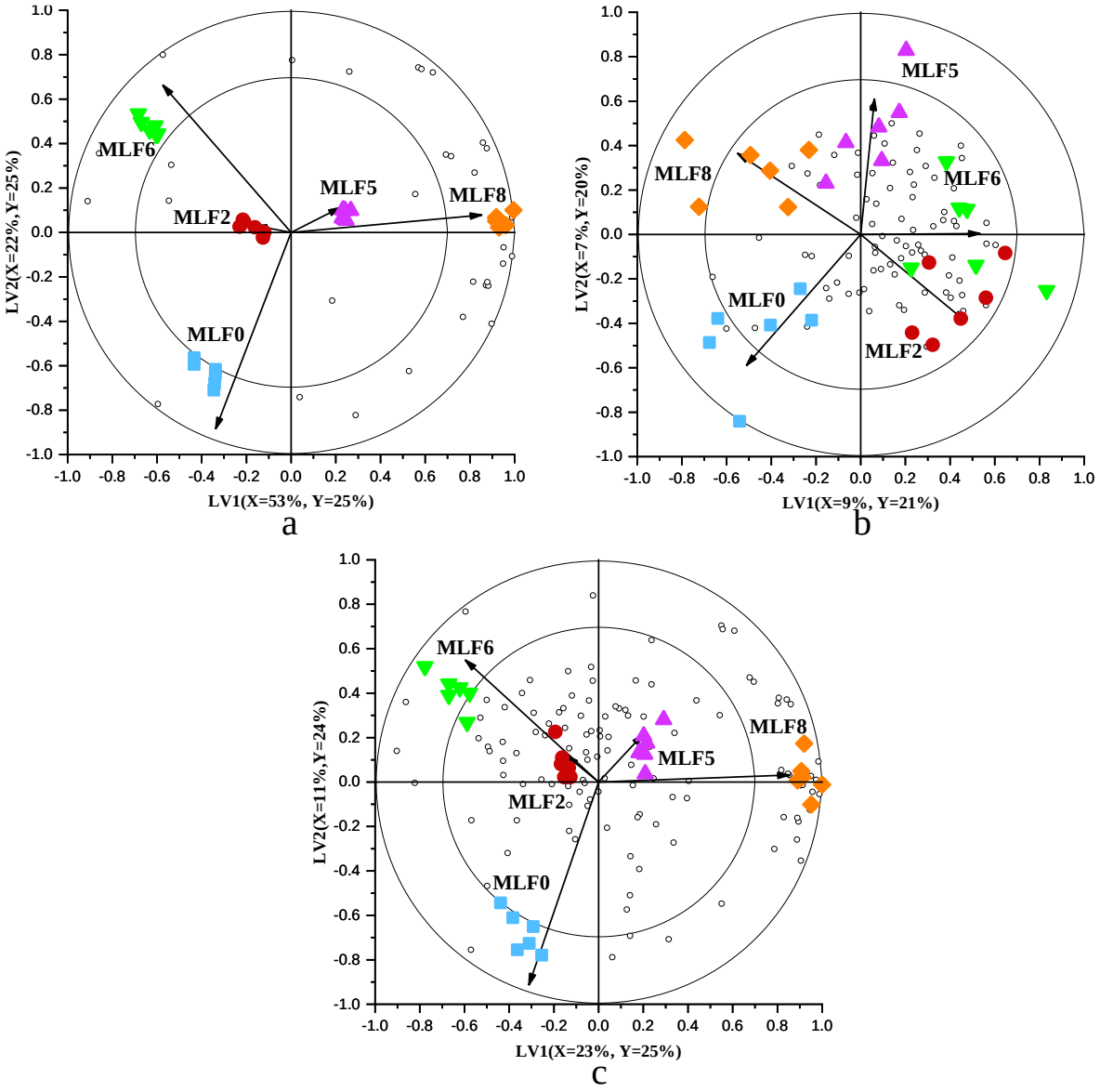

Figure 4.11 PLS-DA bi-plots comparing the lasting effect of different PEF treatments (MLF0, MLF2, MLF5, MLF6, and MLF8) on the final wines after MLF according to (a) phenolic profile, (b) volatile profile, and (c) combined phenolic profile, volatile profile, colour, and oenological properties of fermented grape juice after MAF stage. Classes are represented as coloured solid shapes and metabolites are drawn as open circles. Vectors signify the correlation loadings for the categorical *Y*-variables. The percentages of *X*- and *Y*-variances explained by each latent variable (LV) are specified on the respective axes.

Table 4.6 Discriminant phenolic and volatile compounds, colour, and oenological attributes selected based on VID coefficient of ≥ |0.800| for Merlot wines produced from untreated (MLF0) and PEF-treated (MLF2, 5, 6 and 8) grapes. Phenolic compounds were identified and quantified with standards. The retention index (RI) for volatile compounds is also listed.

VID	Identity	RI	Chemical group
MLF0: Untreated			
0.905	neochlorogenic acid		hydroxycinnamic acid
-0.818	gallic acid		hydroxybenzoic acid
-0.831	trans-p-coumaric acid		hydroxycinnamic acid
MLF2: 33.1 kV/cm, 18.9 kJ/L			
0.917	1-hexanol, 2-ethyl	1534	higher alcohol
0.904	rutin		flavonol
-0.895	trans-resveratrol		stilbene
MLF5: 33.1 kV/cm, 47.3 kJ/L			
0.819	isoquercitrin		flavonol
MLF6: 41.5 kV/cm, 16.5 kJ/L			
0.851	protocatechuic acid		hydroxybenzoic acid
-0.822	*b**		
-0.827	myricetin glucoside		flavonol
-0.864	malvidin 3-*O*-(6-acetyl)-glucoside		anthocyanin
-0.871	*C**		
-0.871	astilbin		flavanonol
-0.872	*a**		
-0.886	malvidin 3-*O*-(6-p-coumaroyl)-glucoside		anthocyanin
-0.895	petunidin 3-*O*-glucoside		anthocyanin
-0.907	piceid		stilbene
-0.931	malvidin 3-*O*-glucoside		anthocyanin
MLF8: 41.5 kV/cm, 49.4 kJ/L			
0.961	TSS		
0.952	caftaric acid		hydroxycinnamic acid
0.944	peonidin 3-*O*-glucoside		anthocyanin
0.938	delphinidin 3-*O*-glucoside		anthocyanin
0.930	cyanidin 3-*O*-glucoside		anthocyanin
0.926	(+)-catechin		flavanol
0.920	trans-coutaric acid		hydroxycinnamic acid
0.913	(-)-epicatechin		flavanol
0.913	myricetin glucoside		flavonol
0.893	*h*°		
0.892	*b**		
0.846	pH		
0.812	petunidin 3-*O*-glucoside		anthocyanin
0.809	piceid		stilbene
0.808	malvidin 3-*O*-(6-p-coumaroyl)-glucoside		anthocyanin
0.802	*L**		
-0.834	syringic acid		hydroxybenzoic acid
-0.893	vanillic acid		hydroxybenzoic acid

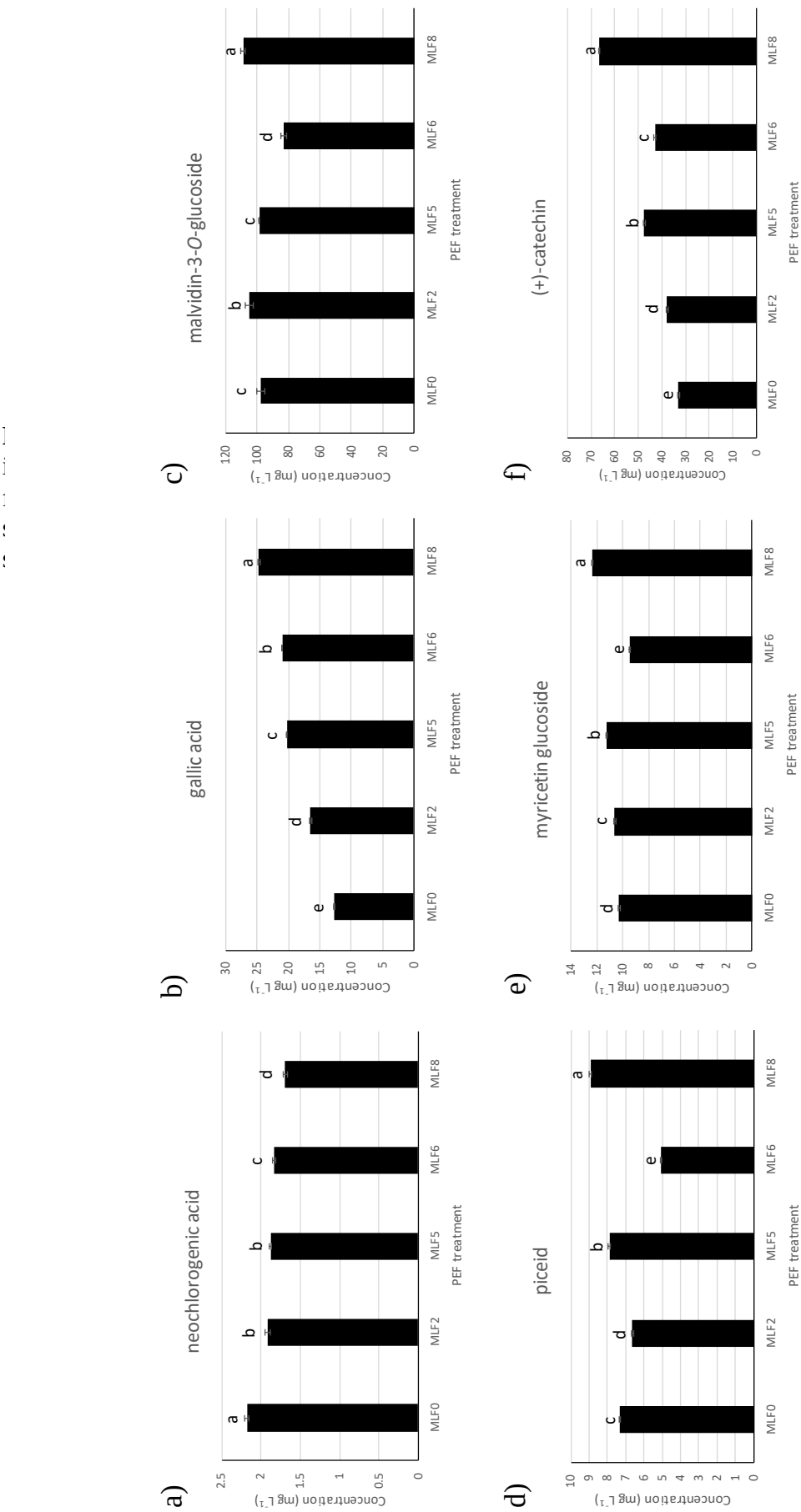

Figure 4.12 Variation in concentration or abundance value of selected discriminant markers in finished Merlot wine samples collected after malolactic fermentation (MLF) of musts from untreated and PEF-treated grapes. Data presented as mean ± standard deviation from six independent measurements (n = 6). Bars with different letters indicate statistically significant differences ($p < 0.05$) between the PEF treatments.

The consequence on the phenolic composition of final wines as a result of increasing electric field strength to 41.5 kV/cm of PEF treatment and total energy up to 49.4 kJ/L when pre-treating the Merlot grapes at the early stage of winemaking was further illustrated in **Figs. 4.12c-f**. In brief, in comparison to MLF0 wine vinified using non PEF-treated grapes, MLF8 wine contained more anthocyanins by up to 37%, both flavanols (+)-catechin and (-)-epicatechin by more than two-folds, the stilbene piceid by 22%, and hydroxycinnamate tartaric esters (caftaric acid and coutaric acid) by 19% to 22%, and myricetin glucoside by 20%. Meanwhile, compared to MLF0, MLF5 have increased anthocyanins by up to 10%, flavonols by more than two-folds, hydroxycinnamic acids by up to 18%, the stilbene piceid by 8%, hydroxybenzoic acids by 7% to 59%, and flavanols from 44% to 60%.

It was also interesting to observe that MLF8 wine attained the lightest (higher L^* value) colour. This inconsistency between the increased concentrations of monomeric anthocyanins but lighter colour could signify the possible interactions between anthocyanins and other phenolic compounds (El Darra, Turk, et al., 2016; Puértolas, Hernández-Orte, et al., 2010). With notably higher concentrations of phenolic compounds (i.e. flavanols, hydroxycinnamic acids, and flavonols) directly involved in copigmentation, complexation, and formation of derived pigments with anthocyanins, MLF8 can have improved colour stability and, thus, may retain this colour longer than other wine samples (Boulton, 2001; Gutiérrez, Lorenzo, & Espinosa, 2005). This can be confirmed through further time storage-stability studies.

Since volatile composition was rather comparable among the wine samples, only one volatile compound 2-ethyl-1-hexanol was selected as distinct marker dominating MLF2 wine. This particular volatile has been detected in aged Merlot wine in a previous study (Zhang et al., 2013). As mentioned in *Section 3.4* regarding MAF process, this distinction in the volatile composition might have come from the differences in precursors extracted at PM and after MAF. Nevertheless, its impact on the overall wine aroma has to be confirmed through further sensory evaluation.

Ultimately, through maceration and both yeast and bacterial fermentations, the findings confirm that the rate of phenolic extraction immediately after PEF treatment is influential on the final wine product. Wines were differentiated according to the specific energies applied at the start of winemaking. PEF treatment at higher total energy (47.25 – 49.40 kJ/L), achieved at different field strengths (33.1 – 41.5 kV/cm), produced wine with increased

phenolic compounds especially anthocyanins, stilbenes, flavonols, flavanols, hydroxycinnamic acids, and hydroxybenzoic acids. On the other hand, wines obtained from grapes PEF-treated at lower total energy (16.47 – 18.90 kJ/L) contained lower concentrations of anthocyanins, flavonols, flavanols, and stilbenes. Among the given PEF conditions, PEF8 (E = 41.5 kV/cm; W = 49.4 kJ/L) treatment on grapes appeared to produce the most distinct wine (MLF8) from the wine vinified using non PEF-treated grapes.

4.4 Conclusion

This study confirmed that the influence of bio-(chemical) reactions during winemaking (maceration and fermentations) dominated over the effect of PEF treatment on musts and wines conducted at commercial scale. Zooming into every winemaking stage, however, revealed the significant impact of varying PEF treatment conditions such as electric field strengths and specific energies on the phenolic and volatile composition and colour of the resulting must and wine samples. Immediately after PEF application, more phenolic compounds and less green odourant 2-hexenal were released compared to the control sample. Juices from grapes treated at higher specific energies (47.25 – 49.40 kJ/L) contained the highest amounts of skin- and seed-bound phenolics such as anthocyanins, flavonols, stilbenes, hydroxycinnamic acids, flavanonol, and flavanols. Simultaneous maceration and alcoholic fermentation strongly modified and developed the phenolic and volatile composition of the fermenting musts. PEF-pre-treated-fermented musts contained more anthocyanins, stilbenes, and flavanols, have more volatile esters, and lower green odourant 1-hexanol. Due to the complexity of maceration and alcoholic fermentation, the distinction between samples of varying specific energies were not apparent at this stage but, ultimately, resurfaced after malolactic fermentation. Wines produced from grapes PEF-treated at higher specific energies at electric field strengths of 33.1 – 41.5 kJ/L retained higher levels of anthocyanins, stilbenes, flavonols, flavanols, hydroxycinnamic acids, and hydroxybenzoic acids. Between the two PEF conditions of comparable high specific energy, the treatment performed at higher electric field strength produced the most distinct wine. This recent advancement on PEF equipment capacities to operate at high electric field strengths generating relatively lower specific energies for commercial practice could potentially enable winemakers to tailor novel wines with enhanced phenolic composition and aroma.

CHAPTER 5

EVOLUTION OF MAJOR PHENOLIC AND VOLATILE COMPOUNDS AS A FUNCTION OF TIME AND TEMPERATURE DURING BOTTLE STORAGE OF MERLOT WINES FROM PEF-TREATED GRAPES: INTEGRATED FINGERPRINTING, PROFILING AND CHEMOMETRICS APPROACH

5.1 Introduction

In *Chapter 4*, finished wines from differently PEF-treated and untreated Merlot grapes were strongly differentiated according to the varying phenolic compositions as a result of their pre-maceration treatment. However, these qualities in the young wines can change over time in bottle storage prior to consumption. During this period, crucial changes in the phenolic and volatile composition affecting the colour, aroma, and taste of the wines occur. Wine evolution during this period can be divided into three phases: maturation, peak quality, and deterioration. During maturation, wine colour, flavour, and stability are expected to refine to reach the peak quality, which is then followed by quality deterioration (Arapitsas, Speri, Angeli, Perenzoni, & Mattivi, 2014). Storage conditions such as time and temperature, together with the initial composition of the wine prior to storage, are important factors that can influence the rate of quality changes (Avizcuri et al., 2016; Mattivi et al., 2015; Scrimgeour et al., 2015). Hence, investigating these factors can determine the stability of the wines fermented from PEF-treated grapes (PEF wines) and might be helpful in establishing appropriate storage conditions to preserve their quality.

A few previous studies have been conducted on PEF wines after malolactic fermentation, which focused on the evolution of the wine phenolic composition and colour during aging in bottles (Puértolas et al., 2009; Puértolas, Saldaña, Condón, et al., 2010) and oak barrels (Puértolas, Saldaña, Álvarez, & Raso, 2010). After 3 months of bottle storage at 18°C, there was no significant changes in the total polyphenol index (TPI, measured using Folin-Ciocalteu colorimeter assay) for Cabernet Sauvignon wines fermented from grapes PEF-treated at an electric field strength of 5 kV/cm and specific energy of 3.67 kJ/kg and was found to be in a similar concentration observed in the control wine (Puértolas et al., 2009). When extending the bottle ageing up to 12 months at 18°C, reduction of TPI in PEF wines was greater than in the control wines; nonetheless, PEF wines still contained a higher TPI (Puértolas, Saldaña, Condón, et al., 2010). With respect to the total monomeric anthocyanins (TMA), flavan-3ols, hydroxycinnamic acids and flavonols, previous study has demonstrated that these compounds were also significantly reduced during bottle ageing, although at different rates compared to the control wine (Puértolas, Saldaña, Condón, et al., 2010). However, because PEF wines contained a higher level of these compounds prior to ageing, TMA concentration in PEF aged wines was eventually found to be similar to the control,

while those non-anthocyanin compounds were in fact higher than control wines upon the completion of ageing process (Puértolas, Saldaña, Condón, et al., 2010). Differences in the rate of phenolic reduction during bottle ageing or storage between PEF wines and control revealed that a PEF pre-treatment applied to grapes at the onset of vinification may play a key role towards modulating the evolution and stability of various chemical constituents important for wines' quality when they are stored in bottle. However, the investigation of these differences in previous studies based solely on the changes of pre-determined phenolic compounds and attributes considerably overlooks the synergistic and cascading effects of a number of phenolic compounds, colour, and volatility of aroma compounds.

The purpose of this study is to describe the overall evolution of wines from PEF-treated Merlot grapes by employing multiplatform analytical approach through combined analysis of phenolic profiles, volatile fingerprints, colour, and oenological attributes, coupled with multivariate data analyses such as principal component analysis (PCA) and partial least square – regression (PLS-R). Important changes over storage time at each storage temperature of 4°C, 25°C, and 45°C for each wine (PEF or no PEF wines) were determined through the selection of discriminant markers.

5.2 Materials and Methods

5.2.1 Grapes and PEF-assisted vinification process

Merlot wines were elaborated according to standard red winemaking procedure in an Auckland-based commercial winery as previously described in *Chapter 4, Section 4.2.1*. A batch of Merlot grapes (*Vitis vinifera* var. Merlot, 5 tonnes) were harvested from Hawke's Bay region (39 °South, 176 °East; North Island, New Zealand). The grapes were harvested at the end of March 2016 at the optimum ripening stage (i.e. 17.80 – 19.80 °Brix; 4.92 – 5.84 g tartaric acid/L; pH 3.48 - 3.56) and transported overnight into the winery for storage (5°C) and vinification. Immediately after destemming and crushing, Pulsed Electric Fields (PEF) were applied onto the must as described in *Chapter 4, Section 4.2.2*. Four different PEF processing conditions (coded as PEF2, 5, 6 and 8) were achieved using the KEA-WEIN electroporation device (Karlsruhe Institute of Technology, Karlsruhe, Germany) connected to a 6-stage Marx generator with a throughput of 500 kg/h, and the capability to generate high intensity electric field strengths (33.1 – 41.5 kV/cm) at a wider range of total energy output (4.70 – 49.40 kJ/L). A batch of untreated (coded as PEF0) grapes was prepared

concomitantly. After PEF processing, all grape musts were fermented into wines under commercial vinification practice involving maceration-alcoholic and malolactic fermentations. Finished wines have an alcohol content of 11.21 to 11.47 ABV. Then, the finished wines were filled and tightly sealed in individual screw capped bottles as described in *Chapter 4, Section 4.2.3*. All wine bottles (325 ml per bottle) were stored at 4 ^{o}C cold room under dark condition until the commencement of the wine storage experiment.

5.2.2 Wine storage experiment under different time and temperature combinations

For each type of wine (PEF0, 2, 5, 6 and 8), a total of 21 bottles of wine were randomly allocated to be stored at three storage temperatures (4, 25 and 45 ^{o}C; 7 bottles per temperature). They were stored upright under dark condition and then each bottle was sampled at predefined time interval. Aliquots were labelled according to the PEF conditions (i.e. P0, P2, P5, P6, and P8 for PEF0, 2, 5, 6, and 8, respectively) and the number of storage days (e.g. D000 and D060 for 0 day/initial sampling and 60 days of storage, respectively). The storage experiment at three different temperatures started on the same day and a batch of wines was sampled on the day before transferring them into the allocated incubators (thereafter referred as "P0D000, P2D000, P5D000, P6D00, and P8D000" samples). These samples were immediately analyzed for their oenological and colour properties. Wines were sampled at specified time intervals which allow the estimation of rate constant for each analyte at different storage temperatures. Wines stored in a 4 ^{o}C temperature-controlled room were sampled at Day 0, 60, 90, and 150. For wines stored at 25 ^{o}C temperature-controlled room, they were sampled at Day 0, 14, 30, 60, 90, and 120; while wines stored at 45 °C incubator (Jeiotech IB-15G, Lab Companion, Seoul, South Korea) were sampled at Day 0, 6, 14, 28, and 56. On the day of sampling, wine bottles were removed from the incubators (especially for 25 and 45 ^{o}C) and placed in 4 ^{o}C temperature-controlled room for no more than 2 h before opening the screw capped bottles. Then four 35-mL aliquots from newly opened bottle were immediately frozen using liquid nitrogen and stored at -80°C until further analysis of phenolic and volatile profiles.

5.2.3 Determination of the oenological properties of stored wines

The remaining wines were analyzed on the day of sampling for total soluble solids (using a refractometer), titratable acidity (using NaOH as titrant to achieve pH 8.2) and pH (using a pH meter) as described earlier in *Chapter 4, Section 4.2.4*. The colour properties of the wine

samples collected at different storage temperature and time combinations were expressed as colour intensity (CI) (measured using a UV/vis spectrophotometer) and colour space values (*L**, *a**, *b**, C*, and H*) using a colorimeter as described in *Chapter 4, Section 4.2.5*. These measurements were done in four replicates.

5.2.4 Determination of anthocyanins and phenolic composition in stored wines

Targeted measurement of 29 phenolic compounds were conducted using the same protocol and HPLC-DAD equipment as detailed in *Chapter 4, Section 4.2.6.* Four wavelengths were used to detect different phenolic classes: 520 nm for anthocyanins, 360 nm for flavonols, 325 nm for hydroxycinnamic acids and stilbenoids, and 280 nm for hydroxybenzoic acids. Phenolic compounds were identified and quantified based on the retention times of the known standards (95% purity) and the external calibration curve of the respective authentic standard. Results were then expressed as mg phenolic per litre of wine.

5.2.5 Determination of the volatile fingerprints for stored wines

Untargeted analysis of the volatile profile of the wine samples were performed according to the volatile extraction and gas chromatography methods conducted in *Chapter 4, Section 4.2.7*. Wine samples were prepared and volatile compounds were extracted through headspace-solid phase micro-extraction (HS-SPME) and analysed by the same gas chromatography system coupled with mass spectrometry detector. GC-MS total ion chromatograms were pre-processed via AMDIS and MPP. Peaks were identified and validated through the following criteria: (a) match and reverse match with the NIST library of not less than 90%; (b) comparison of the experimental retention index (RI) with RI according to literature; and (c) matching retention time with standards for each chemical group.

5.2.6 Multivariate data analysis through Partial Least Squares – Regression (PLS-R)

Data on the oenological properties, colour, phenolic and volatile profiles were pooled together into one data table for multivariate data analysis (MVDA). To investigate the changes as a function of storage time, data sets from samples of the same PEF treatment stored at the same temperature were grouped together to produce 15 data set groups (i.e. 5 PEF treatments X 3 storage temperatures). MVDA of these merged data sets were carried out as specified by Kebede et al. (2015) and Buvé et al. (2018). Using SOLO software

(Version 6.5, 2011, Eigenvector Research, Wenatchee, WA, USA), Principal Component Analysis (PCA) and Partial Least Squares – Regression (PLS-R) were performed. Based on PCA bi-plots, outliers and groupings were detected. Afterwards, PLS-R was conducted to describe the effect of storage time. For PLS-R, the metabolites and storage time were designated as X- and Y-variables, respectively. The optimum number of latent variables (LV) was applied to explain maximum variance/information in the data while maintaining error to a minimum in a model. From this model, PLS-R bi-plots were constructed using Origin Pro 2018 software (Origin Lab Corporation, Northampton, Massachusetts, USA). This is a graphical representation of the differences between samples of varying storage time. To identify metabolites significantly affected by storage time, variable identification coefficients (VID) were calculated as the correlation coefficient of the original *X*-variable (metabolites) to the *Y*-variable (storage time) based on the PLS-model. Metabolites with the minimum VID coefficient of |0.800| were designated as discriminant markers.

5.3 Results and discussion

In this study, the effect of storage times and temperatures on wines from differently PEF-treated and untreated Merlot grapes were extensively examined by observing up to 75 volatile compounds, 29 phenolic compounds, their colour and oenological properties, followed by modelling their changes over storage time through PLS-R, and finally identifying the discriminant compounds and properties considerably affected by both the storage time and temperature.

5.3.1 Visualisation on the evolution of the oenological, phenolic and volatile fingerprints for the bottled PEF wines at three temperatures as a function of storage time

To best visualise the evolution of the bottled wines at three different storage temperatures, PLS-R bi-plots were constructed using two latent variables (LVs) (**Fig. 5.1 – 5.5**). In all fifteen bi-plots, storage time has clearly separated and ordered the samples from the initial (left side of bi-plot) to the last sampling point (right side of bi-plot). This is further indicated by the vectors which represent the correlation loading for *Y*-variable (storage time). The long vectors indicate that the two represented LVs on each bi-plot explained higher percentage of *Y*-variation (Wibowo, Grauwet, Kebede, Hendrickx, & Van Loey, 2015). This variation is driven by the significant changes in the concentrations of the metabolites which are

represented as the open circles on the bi-plots. The relationship between the metabolites and storage time can be interpreted based on their projection relative to the vector and the ellipses, where inner and outer ellipses signify correlation coefficients of 70% and 100%, respectively. Those metabolites positioned beyond the inner ellipse and on the direction of the vector increased as a function of storage time. On the other hand, those metabolites found beyond the inner ellipse but directed opposite the vector decreased during storage. Meanwhile, metabolites inside the inner ellipse can be considered somewhat stable during storage. In all stored wines, most metabolites reduced over time.

To identify these metabolites affected by storage time at each storage temperature in each PEF condition, VID coefficients of the attributes were calculated. **Table 5.1-5.5** listed metabolites significantly increasing and decreasing as a function of storage time on stored wines from differently PEF-treated and untreated Merlot grapes.

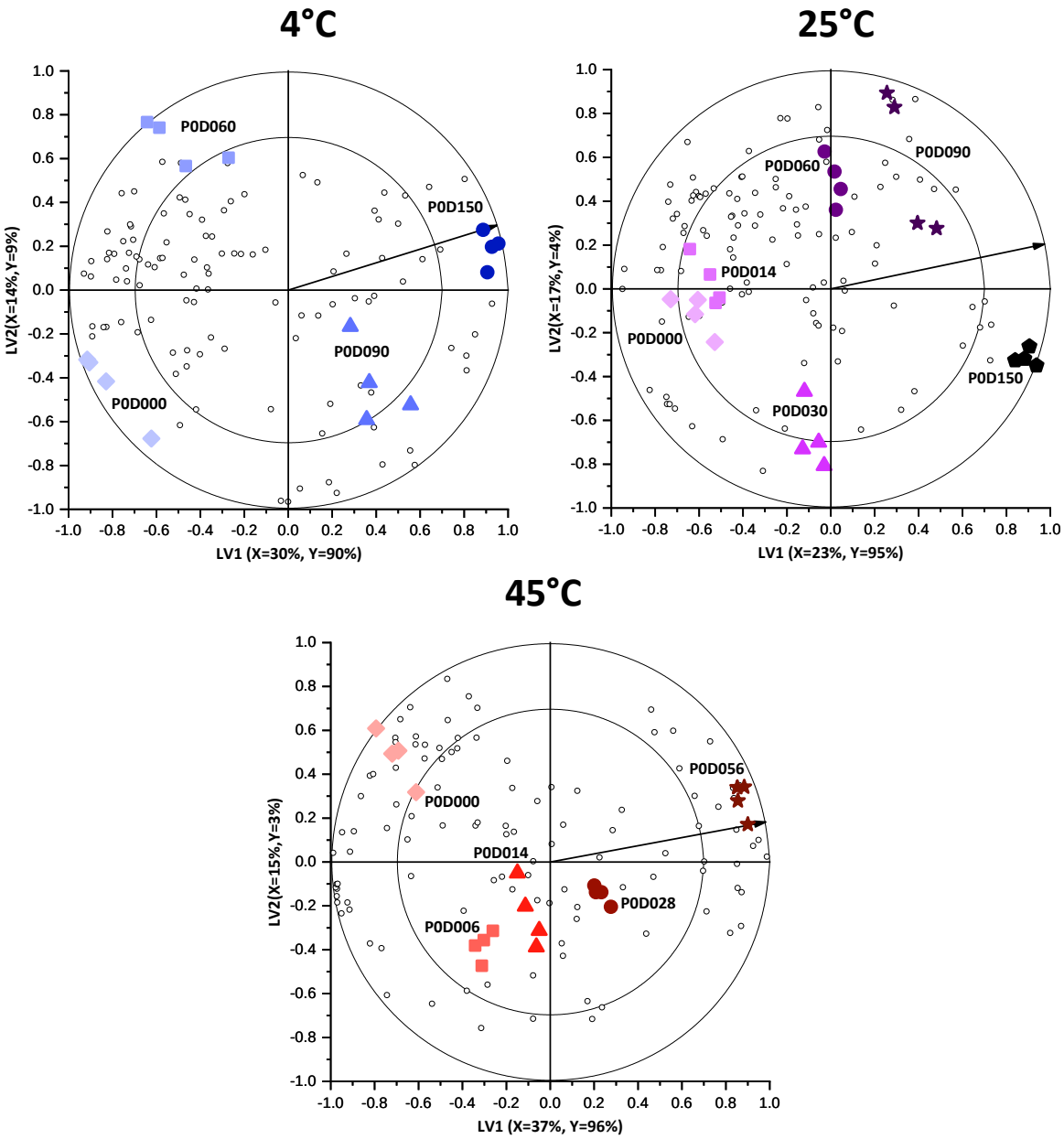

Figure 5.1 PLS-R bi-plots illustrating the changes as a function of storage time on the phenolic profile, volatile fingerprint, colour, and basic oenological properties of Merlot wines from untreated (PEF0) grapes. Classes based on storage time are represented as coloured solid shapes and labelled (e.g. P0D000) according to PEF treatment (e.g. P0 for PEF0) and number of days (e.g. D000 for day 0). Metabolites are drawn as open circles. Vectors signify the correlation loadings for the categorical Y-variables. The percentages of X- and Y-variances explained by each latent variable (LV) are specified on the respective axes.

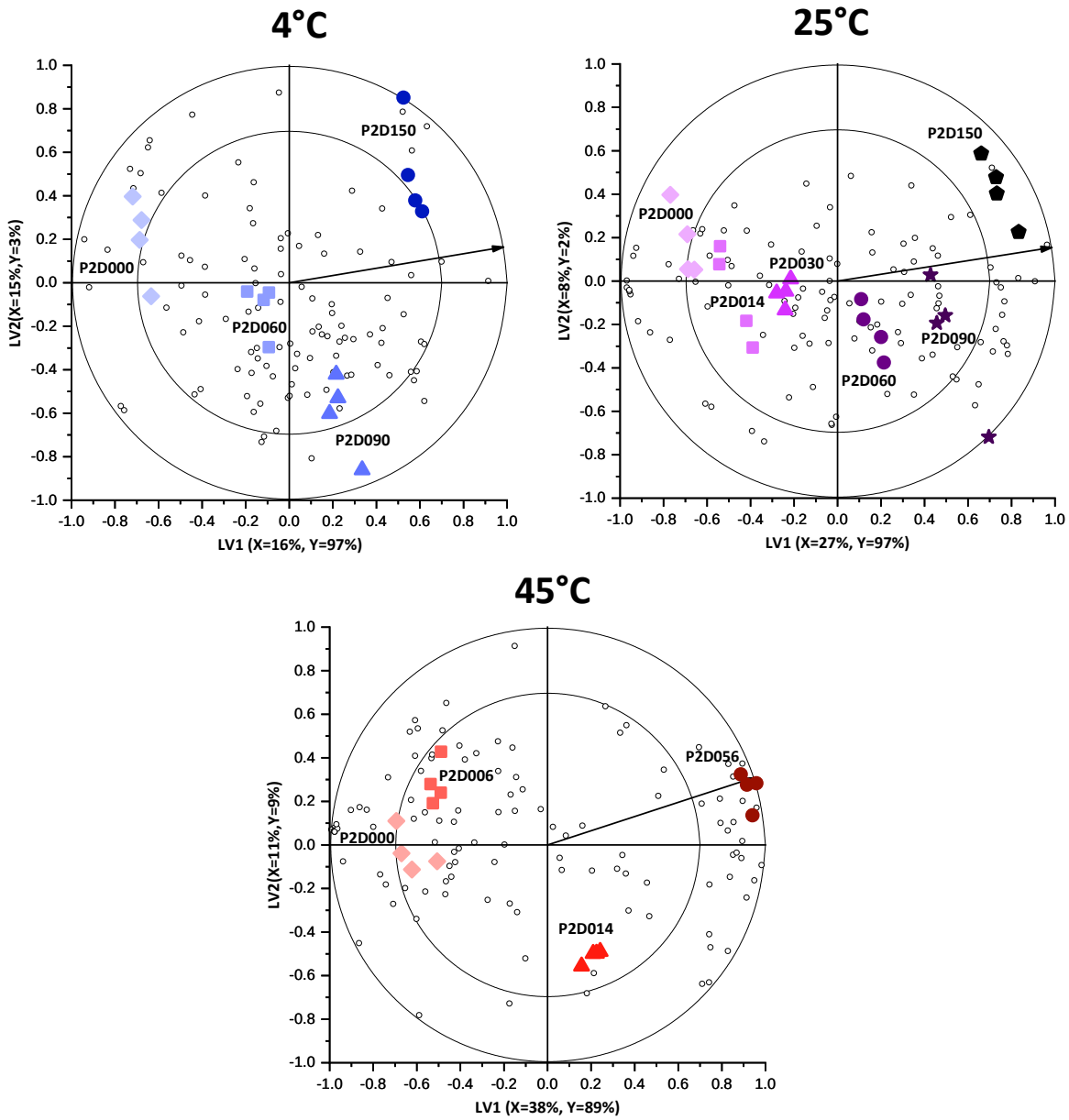

Figure 5.2 PLS-R bi-plots illustrating the changes as a function of storage time on the phenolic profile, volatile fingerprint, colour, and basic oenological properties of Merlot wines from PEF2-treated grapes. Classes based on storage time are represented as coloured solid shapes and labelled (e.g. P2D000) according to PEF treatment (e.g. P2 for PEF2) and number of days (e.g. D000 for day 0). Metabolites are drawn as open circles. Vectors signify the correlation loadings for the categorical Y-variables. The percentages of X- and Y-variances explained by each latent variable (LV) are specified on the respective axes.

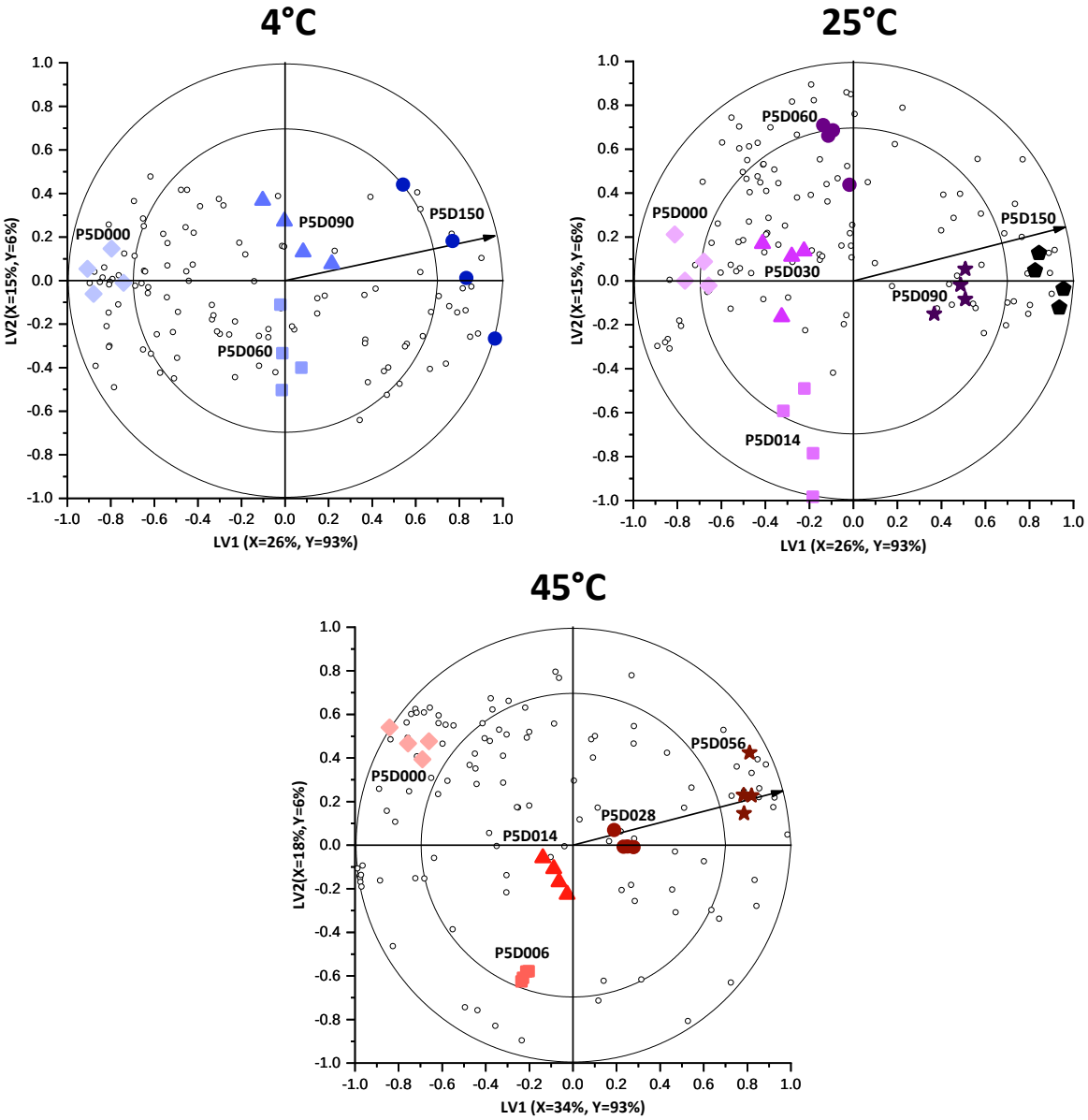

Figure 5.3 PLS-R bi-plots illustrating the changes as a function of storage time on the phenolic profile, volatile fingerprint, colour, and basic oenological properties of Merlot wines from PEF5-treated grapes. Classes based on storage time are represented as coloured solid shapes and labelled (e.g. P5D000) according to PEF treatment (e.g. P5 for PEF5) and number of days (e.g. D000 for day 0). Metabolites are drawn as open circles. Vectors signify the correlation loadings for the categorical Y-variables. The percentages of X- and Y-variances explained by each latent variable (LV) are specified on the respective axes.

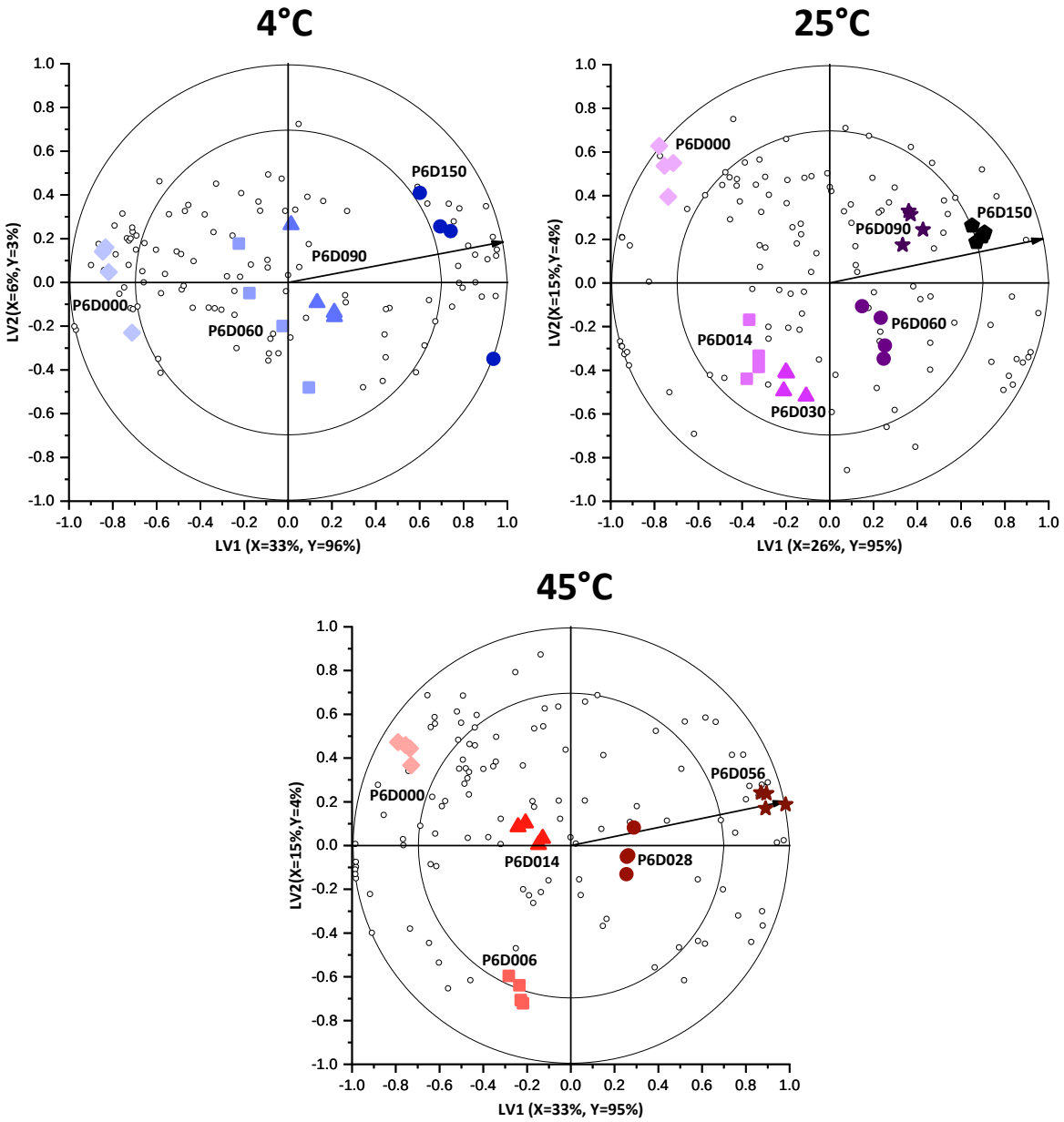

Figure 5.4 PLS-R bi-plots illustrating the changes as a function of storage time on the phenolic profile, volatile fingerprint, colour, and basic oenological properties of Merlot wines from PEF6-treated grapes. Classes based on storage time are represented as coloured solid shapes and labelled (e.g. P6D000) according to PEF treatment (e.g. P6 for PEF6) and number of days (e.g. D000 for day 0). Metabolites are drawn as open circles. Vectors signify the correlation loadings for the categorical Y-variables. The percentages of X- and Y-variances explained by each latent variable (LV) are specified on the respective axes.

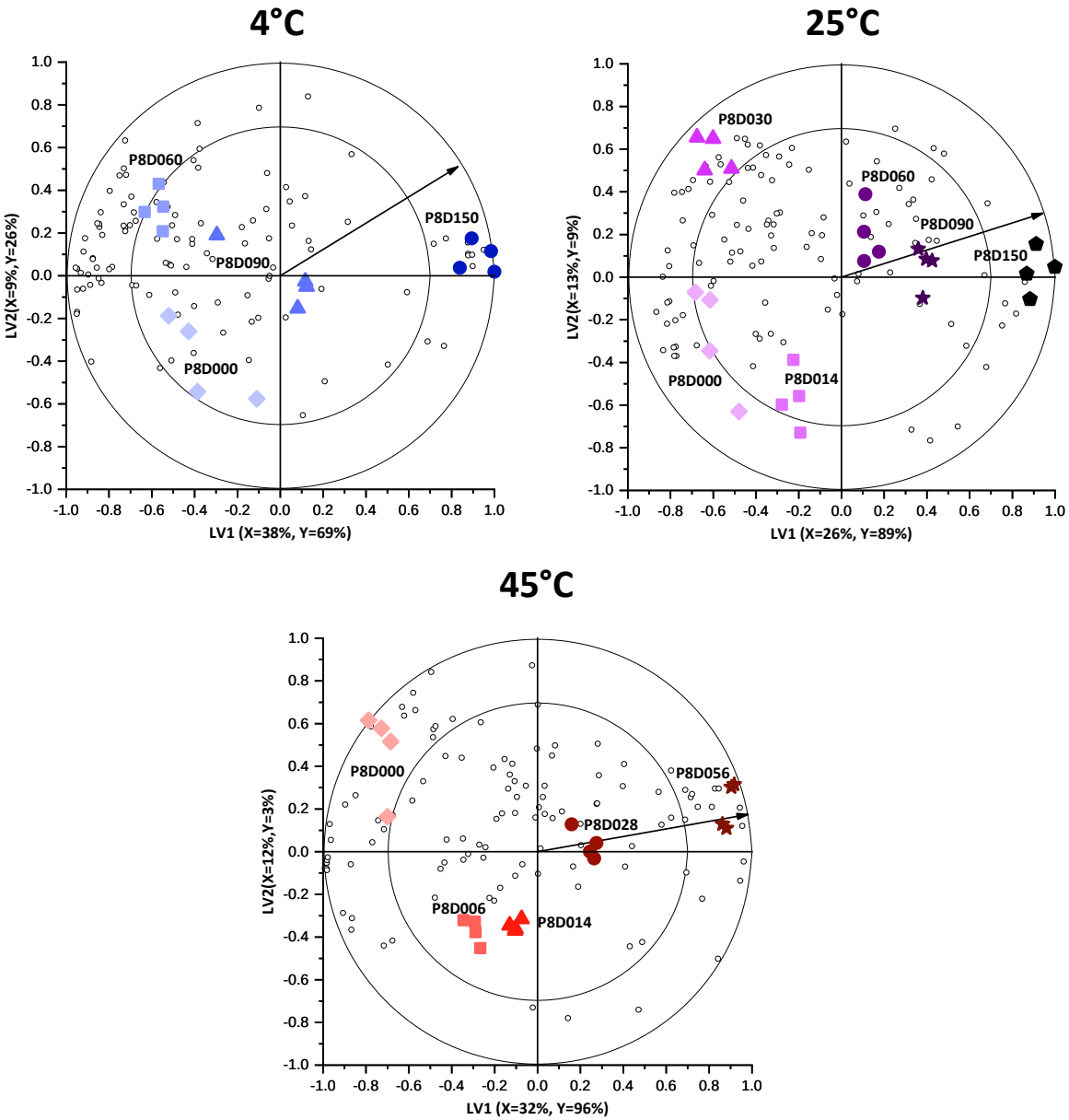

Figure 5.5 PLS-R bi-plots illustrating the changes as a function of storage time on the phenolic profile, volatile fingerprint, colour, and basic oenological properties of Merlot wines from PEF8-treated grapes. Classes based on storage time are represented as coloured solid shapes and labelled (e.g. P8D000) according to PEF treatment (e.g. P8 for PEF8) and number of days (e.g. D000 for day 0). Metabolites are drawn as open circles. Vectors signify the correlation loadings for the categorical Y-variables. The percentages of X- and Y-variances explained by each latent variable (LV) are specified on the respective axes.

Table 5.1. Discriminant phenolic and volatile compounds, colour, and oenological attributes significantly changed as a function of time at each storage temperature (4, 25, and 45°C) selected based on VID coefficient of ≥ |0.800| for wines from untreated Merlot grapes (PEF0). Phenolic compounds were identified and quantified with standards. The retention index (RI) for volatile compounds is also listed.

4°C			25°C			45°C		
VID	**Identity**	**RI**	**VID**	**Identity**	**RI**	**VID**	**Identity**	**RI**
0.894	gallic acid					0.983	piceid	
0.891	*trans*-resveratrol					0.954	syringic acid	
						0.927	furfural	1639
						0.897	diethyl butanedioate	1846
						0.867	(4S,5R)-4-methyl-5-(3-methyl-2-buten-1-yl)dihydro-2(3H)-furanone	1058
						0.865	ethyl 2-hydroxy-3-methylbutanoate	1588
						0.859	linalool 3,7-oxide	1197
						0.844	pH	
						0.834	trans-coutaric acid	
						0.829	*trans*-resveratrol	
-0.844	*a**		-0.815	malvidin-3-(6-*p*-coumaroyl)-glucoside		-0.809	(E)-astringin	
-0.849	2-phenylethyl acetate	1993	-0.826	peonidin 3-*O*-glucoside		-0.810	TA	
-0.857	*C*		-0.828	malvidin 3-*O*-glucoside		-0.822	linalool	1708
-0.865	1-heptanol	1610	-0.895	malvidin 3-(6-acetyl)-glucoside		-0.859	caftaric acid	
-0.894	ethyl butanoate	1095	-0.906	*b**		-0.872	citronellol	1925
-0.906	acetic acid	1643	-0.933	isorhamnetin glucoside		-0.900	2-phenylethyl acetate	1993
-0.916	*b**					-0.926	CI	
						-0.934	astilbin	
						-0.939	rutin	
						-0.972	cyaniding-3-*O*-glucoside	
						-0.973	malvidin-3-(6-*p*-coumaroyl)- glucoside	
						-0.977	neochlorogenic acid	
						-0.978	malvidin-3-(6-acetyl)-glucoside	
						-0.979	delphinidin-3-*O*-glucoside	
						-0.984	petunidin-3-*O*-glucoside	
						-0.984	malvidin-3-*O*-glucoside	
						-0.985	peonidin-3-*O*-glucoside	

Table 5.2. Discriminant phenolic and volatile compounds, colour, and oenological attributes significantly changed as a function of time at each storage temperature (4, 25, and 45°C) selected based on VID coefficient of ≥ |0.800| for wines from PEF2-treated Merlot grapes. Phenolic compounds were identified and quantified with standards. The retention index (RI) for volatile compounds is also listed.

4°C			25°C			45°C		
VID	**Identity**	**RI**	**VID**	**Identity**	**RI**	**VID**	**Identity**	**RI**
0.903	protocatechuic acid		0.974	gallic acid		0.975	diethyl butanedioate	1846
			0.829	ethyl 2-methyl-butanoate	1113	0.953	pH	
						0.944	nonanal	1555
						0.917	(4S,5R)-4-methyl-5-(3-methyl-2-buten-1-yl)dihydro-2(3H)-furanone	1058
						0.908	piceid	
						0.893	ethyl 2-furancarboxylate	1801
						0.884	3-hydroxy-2,2,4-trimethylpentyl 2-methylpropanoate	2026
						0.881	2,2,4-trimethyl-1,3-pentanediol diisobutyrate	2033
						0.867	linalool 3,7-oxide	1197
						0.860	syringic acid	
						0.859	trans-*p*-coumaric acid	
						0.849	*trans*-resveratrol	
						0.832	octanol	1720
						0.826	butyl ethyl butanedioate	1956
						0.816	ethyl 3-methylbutyl butanedioate	2051
-0.841	*a**		-0.879	isorhamnetin glucoside		-0.820	2-phenylethyl acetate	1993
-0.853	*C*		-0.909	malvidin-3-(6-*p*-coumaroyl) glucoside		-0.863	linalool	1708
-0.897	*b**		-0.926	myricetin glucoside		-0.929	caftaric acid	
-0.911	kaempferol glucoside		-0.930	cyanidin-3-*O*-glucoside		-0.938	CI	
			-0.954	delphinidin-3-*O*-glucoside		-0.938	malvidin-3-(6-acetyl)-glucoside	
			-0.958	malvidin-3-*O*-glucoside		-0.939	malvidin-3-(6-*p*-coumaroyl) glucoside	
			-0.958	petunidin-3-*O*-glucoside		-0.951	delphinidin-3-*O*-glucoside	
			-0.965	malvidin-3-(6-acetyl)-glucoside		-0.953	malvidin-3-*O*-glucoside	
			-0.968	peonidin-3-*O*-glucoside		-0.953	rutin	
						-0.954	petunidin-3-*O*-glucoside	
						-0.954	peonidin-3-*O*-glucoside	
						-0.967	cyanidin-3-*O*-glucoside	

Table 5.3. Discriminant phenolic and volatile compounds, colour, and oenological attributes significantly changed as a function of time at each storage temperature (4, 25, and 45°C) selected based on VID coefficient of ≥ |0.800| for wines from PEF5-treated Merlot grapes. Phenolic compounds were identified and quantified with standards. The retention index (RI) for volatile compounds is also listed.

4°C			25°C			45°C		
VID	**Identity**	**RI**	**VID**	**Identity**	**RI**	**VID**	**Identity**	**RI**
0.908	ethyl gallate		0.925	syringic acid		0.977	piceid	
						0.938		
							(4S,5R)-4-methyl-5-(3-methyl-2-	
0.856	gallic acid		0.896	caffeic acid			buten-1-yl)dihydro-2(3H)-furanone	1058
0.848	caffeic acid		0.884	gallic acid		0.936	syringic acid	
0.818	(+)-catechin		0.873	ethyl gallate		0.905	diethyl butanedioate	1846
0.810	astilbin		0.861	diethyl butanedioate	1846	0.889	1-octanol	1720
0.808	caftaric acid					0.879	pH	
						0.867	dipropyl butanedioate	1105
						0.817	linalool 3,7-oxide	1197
						0.805	ethyl 3-methyl-butanoate	1136
						0.801	ethyl 2-hydroxy-3-methylbutanoate	1588
-0.801	ethyl 3-hydroxy-butanoate	1687	-0.808	delphinidin-3-*O*-glucoside		-0.810	citronellol	1925
-0.805	ethyl 2-hydroxy-4-methyl-pentanoate	1712	-0.811	malvidin-3-(6-*p*-coumaroyl) glucoside		-0.827	linalool	1708
-0.819	3-methyl-1-pentanol	1462	-0.819	cyanidin-3-*O*-glucoside		-0.898	caftaric acid	
-0.821	octanol	1720	-0.878	petunidin-3-*O*-glucoside		-0.899	rutin	
-0.832	methoxyacetic acid, 3-methylbutyl ester		-0.879	peonidin-3-*O*-glucoside		-0.966	malvidin-3-(6-*p*-coumaroyl) glucoside	
-0.832	pH		-0.893	malvidin-3-*O*-glucoside		-0.981	malvidin 3-(6-acetyl)-glucoside	
-0.833	*a**		-0.931	malvidin-3-(6-acetyl)-glucoside		-0.987	petunidin-3-*O*-glucoside	
-0.836	*C*					-0.988	malvidin-3-*O*-glucoside	
-0.837	2-heptanol	1450				-0.988	delphinidin-3-*O*-glucoside	
-0.846	protocatechuic acid					-0.990	peonidin-3-*O*-glucoside	
-0.866	3-methyl-butanoate	1857				-0.992	cyanidin-3-*O*-glucoside	
-0.893	(E)-astringin							
-0.906	*b**							

Table 5.4. Discriminant phenolic and volatile compounds, colour, and oenological attributes significantly changed as a function of time at each storage temperature (4, 25, and 45°C) selected based on VID coefficient of ≥ |0.800| for wines from PEF6-treated Merlot grapes. Phenolic compounds were identified and quantified with standards. The retention index (RI) for volatile compounds is also listed.

4°C			25°C			45°C		
VID	Identity	RI	VID	Identity	RI	VID	Identity	RI
0.961	gallic acid		0.920	syringetin glucoside		0.967	piceid	
0.956	caffeic acid		0.917	kaempferol glucoside		0.936	syringic acid	
0.948	syringetin glucoside		0.857	*trans*-coutaric acid		0.932	dipropyl butanedioate	1105
0.939	(+)-catechin		0.843	gallic acid		0.914	(4S,5R)-4-methyl-5-(3-methyl-2-buten-1-yl)dihydro-2(3H)-furanone	1058
0.928	protocatechuic acid		0.815	linalool	1708	0.903	linalool 3,7-oxide	1197
0.915	myricetin glucoside		0.814	piceid		0.846	1-octanol	1720
0.875	rutin		0.812	quercetin		0.838	diethyl butanedioate	1846
0.861	*trans*-resveratrol					0.825	trans-*p*-coumaric acid	
0.852	quercetin					0.822	protocatechuic acid	
0.832	neochlorogenic acid					0.819	*trans*-coutaric acid	
0.806	piceid							
-0.830	isobutyl acetate	1064	-0.801	isorhamnetin glucoside		-0.828	2-phenylethyl acetate	1993
-0.833	acetic acid	1643	-0.814	neochlorogenic acid		-0.835	linalool	1708
-0.848	2-phenylethyl acetate	1993	-0.874	*b**		-0.939	cyanidin-3-*O*-glucoside	
-0.889	ethyl butanoate	1095	-0.903	*C*		-0.955	caftaric acid	
-0.982	*a**		-0.904	*a**		-0.975	malvidin-3-(6-*p*-coumaroyl)-glucoside	
-0.988	*C*		-0.963	malvidin-3-(6-*p*-coumaroyl)-glucoside		-0.983	malvidin-3-(6-acetyl)-glucoside	
			-0.964	peonidin-3-*O*-glucoside		-0.986	delphinidin-3-*O*-glucoside	
			-0.977	malvidin-3-*O*-glucoside		-0.991	petunidin-3-*O*-glucoside	
			-0.982	malvidin-3-(6-acetyl)-glucoside		-0.993	peonidin-3-*O*-glucoside	
			-0.985	petunidin-3-*O*-glucoside		-0.993	malvidin-3-*O*-glucoside	
			-0.987	delphinidin-3-*O*-glucoside				

Table 5.5. Discriminant phenolic and volatile compounds, colour, and oenological attributes significantly changed as a function of time at each storage temperature (4, 25, and 45°C) selected based on VID coefficient of ≥ |0.800| for wines from PEF8-treated Merlot grapes. Phenolic compounds were identified and quantified with standards. The retention index (RI) for volatile compounds is also listed.

4°C			25°C			45°C		
VID	Identity	RI	VID	Identity	RI	VID	Identity	RI
0.946	gallic acid		0.833	gallic acid		0.962	syringic acid	
0.887	*h*°					0.951	caffeic acid	
0.884	caffeic acid					0.925	piceid	
0.875	trans-coutaric acid					0.872	linalool 3,7-oxide	1197
0.871	myricetin glucoside					0.855	dipropyl butanedioate	1105
0.869	*L*					0.831	(4S,5R)-4-methyl-5-(3-methyl-2-buten-1-yl)dihydro-2(3H)-furanone	1058
0.824	2,2,4-trimethyl-1,3-pentanediol diisobutyrate	2033						
-0.810	syringetin-glucoside		-0.802	*h*°		-0.814	citronellol	1925
-0.811	hexyl acetate	1404	-0.811	*a**		-0.867	linalool	1708
-0.817	peonidin 3-o-glucoside		-0.816	2-phenylethyl acetate	1993	-0.872	trans-fertaric acid	
-0.818	quercetin dihydrate		-0.821	*C*		-0.894	caftaric acid	
-0.818	malvidin-3-(6-acetyl)glucoside		-0.828	petunidin-3-*O*-glucoside		-0.903	neochlorogenic acid	
-0.825	ethyl butanoate	1095	-0.834	peonidin-3-*O*-glucoside		-0.933	(+)-catechin	
-0.850	isobutyl acetate	1064	-0.838	*L*		-0.949	astilbin	
-0.859	(E)-astringin		-0.844	malvidin-3-*O*-glucoside		-0.952	rutin	
-0.864	astilbin		-0.844	malvidin-3-(6-*p*-coumaroyl)-glucoside		-0.975	malvidin-3-(6-*p*-coumaroyl)-glucoside	
-0.890	malvidin-3-*O*-glucoside		-0.881	*b**		-0.980	malvidin-3-(6-acetyl)-glucoside	
-0.901	delphinidin-3-*O*-glucoside		-0.889	malvidin-3-(6-acetyl)-glucoside		-0.983	delphinidin-3-*O*-glucoside	
-0.901	cyanidin-3-*O*-glucoside					-0.983	petunidin-3-*O*-glucoside	
-0.906	petunidin-3-*O*-glucoside					-0.985	malvidin-3-*O*-glucoside	
-0.928	kaempferol glucoside					-0.986	peonidin-3-*O*-glucoside	
-0.958	*a**					-0.994	cyanidin-3-*O*-glucoside	
-0.960	*b**							
-0.962	*C*							

5.3.2 Key changes in the colour, oenological, phenolic and volatile fingerprints for the bottled PEF wines at three different storage temperatures as a function of time

The effect of storage time and temperature common to all wines from untreated and PEF-treated grapes can be identified through similar key trends among the markers across the wine samples which are discussed in *Sections 3.2.1 to 3.2.4*. Discriminant markers distinct to certain wines stemming from the differences in the initial composition a result of PEF-pre-treatment are also discussed in *Section 3.2.5*.

5.3.2.1 *Increases in the hydroxycinnamic and hydroxybenzoic acids over storage time*

For all storage temperatures, phenolic acids (i.e. hydroxycinnamic and hydroxybenzoic acids) increased over time for all PEF wines. The higher concentration of hydroxycinnamic acids can be attributed to the hydrolysis of tartaric esters present in the young wines into their corresponding free acids such as caffeic acid and coumaric acids from caftaric acid and coutaric acid, respectively (Agazzi et al., 2018; Arapitsas et al., 2014; Oliveira, Barros, Silva Ferreira, & Silva, 2015). This trend has been observed in red wines from grapes PEF-treated (Puértolas, Saldaña, Condón, et al., 2010) or not (Agazzi et al., 2018; Monagas, Bartolomé, & Gómez-Cordovés, 2005). The surge in hydroxybenzoic acids, particularly gallic acid, protocatechuic acid, and syringic acid, may have resulted from the cleavage of anthocyanins during wine storage (e.g. malvidin 3-*O*-glucoside producing syringic acid) (C. M. Oliveira et al., 2015). Also, the hydrolysis of galloylated tannins in the wines may have contributed to gallic acid concentrations (Cassino et al., 2019).

5.3.2.2 *Decreases in the anthocyanins and colour properties over storage time*

A reduction in the anthocyanins accompanied by the dulling of colour over storage time, particularly at higher storage temperatures was expected. Apart from experiencing degradation, oxidation, and cleavage into hydroxybenzoic acids, monomeric anthocyanins can participate in reactions contributing to the colour stability of the wine including self-association, copigmentation, polymerisation, and formation of new pigments, which has been reported by several authors on storage of red wines (Avizcuri et al., 2016; González-Sáiz et al., 2014; Ivanova, Vojnoski, & Stefova, 2012; Petrozziello et al., 2018). This changes involving anthocyanins explains the shift from red-purple colour imparted by the monomeric anthocyanins in the young wine to the brick red hues resulting from a

combination of new pigments and complexes with anthocyanins (Avizcuri et al., 2016; González-Sáiz et al., 2014).

5.3.2.3 *Reduction of esters and acetates volatiles over storage time*

After 180 days of storage at 4°C, the loss of esters and acetates is among the most significant changes in most wines except from the PEF2-treated grapes. PEF0 and PEF6 wines lost both ethyl butaboate and 2-phenylethyl acetate. PEF8 also lost ethyl butanoate, along with two acetates (i.e. hexyl acetate and isobutyl acetate). PEF5 wine has significantly decreased concentrations of four esters unique from the markers of other wines. These include ethyl-3-hydroxy butanoate, ethyl-2-hydroxy-4-methyl pentanoate, 3-methylbutyl methoxyacetate, and 3-methyl butanoate. In young wines, the presence of these volatile compounds are considered desirable as they contribute towards the fresh and fruity aroma owing to the excess production during yeast fermentation. However, their gradual hydrolysis during aging is unavoidable. Eventually, ester and acetate concentrations reduce to reach a balance with respective alcohols and fatty acids resulting to the loss of freshness in stored or aged wines (Antalick, Perello, & de Revel, 2014; Pérez-Prieto, López-Roca, & Gómez-Plaza, 2003; Rapp & Mandery, 1986).

5.3.2.4 *Wine constituents identified to be temperature sensitive under extreme storage condition of 45ºC*

Elevated temperatures during storage is expected to accelerate various chemical reactions in wines involving colour change, loss of phenolic and anthocyanin compounds, reducing volatile compounds that contribute to the freshness of wine aroma and formation of new volatile compounds (Czibulya, Kollár, Pour Nikfardjam, & Kunsági-Máté, 2012; Robinson et al., 2010; Scrimgeour et al., 2015). Therefore, the ideal storage temperature for red wines intended for consumption is recommended at around 15-20°C (Scrimgeour et al., 2015). In this study, ambient storage temperature is represented by 25°C, while both the lowest (4°C) and highest (45°C) storage temperatures can be considered extreme.

In the present study, wine storage at 45°C for up to 56 days has affected different classes of phenolic and volatile compounds. With respect to phenolics, piceid was found to increased, while caftaric acid and rutin decreased over time. The increase in piceid during storage is poorly understood. It can be speculated to be caused by the isomerization of *cis*-piceid to its

trans- form (Clare et al., 2005; Lamuela-Raventós, Romero-Pérez, Waterhouse, & de la Torre-Boronat, 1995). Moreover, the presence of trace activity of glycosyltransferase enzymes in wine capable of re-glycosylation of resveratrol into piceid during storage may be possible (Lepak, Gutmann, Kulmer, & Nidetzky, 2015; Ono et al., 2010; Robinson et al., 2014). In the case of caftaric acid reduction, its hydrolysis into its corresponding caffeic acid is expected to be a slow process in conventional ageing and storage of red wines. However, this reaction has been demonstrated to accelerate in high temperatures (Arapitsas et al., 2014; Gutiérrez et al., 2005; Panceri & Bordignon-Luiz, 2017). The loss of rutin might be due to its active copigmentation with anthocyanins (Boulton, 2001). Heras-Roger, Alonso-Alonso, Gallo-Montesdeoca, Díaz-Romero, and Darias-Martín (2016) has demonstrated high correlation of rutin concentration on the copigmentation factor in the overall colour of red wines.

With respect to volatile compounds, wines stored in the extreme high temperature in this study were characterized by an increase of the specific ester diethyl butanedioate/diethyl succinate, increase of the furanic compound (4S,5R)-4-Methyl-5-(3-methyl-2-buten-1-yl)dihydro-2(3H)-furanone, increase of the terpene oxide linalool 3,7-oxide (ethenyltetrahydro-2H-pyran), and the decrease of the latter's respective terpene alcohol linalool. Diethyl butanedioate/diethyl succinate has been recorded to increase at 40°C in Aglianco del Vulture red wine (D'Auria, Emanuele, & Racioppi, 2009; Shinohara & Watanabe, 1981) and is among the esters listed to increase during ageing (Linsenmeier, Rauhut, & Sponholz, 2010). Furanic compounds are produced through nonenzymatic browning reactions favoured at high storage temperature. These reactions, namely caramelisation and Maillard reaction, form a variety furanic compounds which may or may not be desired depending on the wine product (Ho, Hogg, & Silva, 1999; Mayr et al., 2015; Pereira, Albuquerque, Cacho, & Marques, 2013; Scrimgeour et al., 2015). Linalool 3,7-oxide is a product of the degradation of linalool, which has been demonstrated to develop at 40°C in Merlot and Cabernet Sauvignon wines (Robinson et al., 2010; Silva Ferreira, Guedes de Pinho, Rodrigues, & Hogg, 2002; Williams, Strauss, & Wilson, 1980).

5.3.2.5 *Effect of PEF pre-treatments on Merlot grapes on the evolution of wine constituents during storage*

As demonstrated in *Chapter 4*, the finished wines fermented from differently PEF-treated Merlot grapes varied mostly in their phenolic composition upon completion of the vinification process (see **Table 4.5**). This variation seemed to affect the evolution of some phenolic and volatile markers distinct to a certain wine sample during the subsequent storage. Prolonged storage at 4°C for wines vinified using grapes treated with PEF at the highest specific energy (PEF8: 49.40 kJ/L) have significantly reduced amount of eleven phenolic compounds classified as anthocyanins, flavonols, a flavanonol, and a stilbene (**Table 5.5**). With higher initial content of anthocyanins throughout the winemaking process, wines from PEF8-treated grapes lost significant amount of anthocyanins during storage, which has not been observed on other wine samples (**Table 5.1 to 5.5**). As discussed, this reduction could be due to degradation, oxidation, and cleavage self-association, and also copigmentation, polymerisation, and formation of new pigments (Avizcuri et al., 2016; González-Sáiz et al., 2014; Ivanova et al., 2012; Petrozziello et al., 2018). This decrease is aligned with the observation from the study of Puértolas, López, et al. (2010), where a faster reduction rate of monomeric anthocyanins in PEF wines has been reported; although, no significant differences was observed between wines after storage. In the same study, the rates of reduction for the other non-anthocyanin compounds were found to be either faster or slower in PEF wines compared to the control wines. However, PEF wines retained higher amounts after storage. Hence, kinetic modelling of these compounds is needed as future investigation to appropriately compare their storage and thermal stability in the wines tested in this study.

Prolonged storage at 25°C has resulted in an increment of phenolic acids in PEF stored wines but not in the control wine (**Table 5.1 to 5.5**). This might be due to higher concentration of the precursors present in PEF wines such as the tartaric esters hydrolysed into free hydroxycinnamic acids, and anthocyanins cleaved into hydroxybenzoic acids during the winemaking process (as discussed in *Section 3.2.1*).

As listed on **Table 5.1 to 5.5**, more volatile markers distinguished the wines stored at 45°C: 1) citronellol decreased in stored wines from grapes untreated and PEF-treated at high specific energies (PM5 and PM8); (2) 2-phenylethyl acetate decreased in stored wines from grapes untreated and PEF-treated at low specific energies (PM2 and PM6); and (3) more

furan compound marker in stored wines from untreated and low intensity PEF-treated (PEF2) grapes. The decrease in citronellol and 2-phenylethyl acetate could be due to increased rate of hydrolysis during storage (Antalick et al., 2014; Rapp & Mandery, 1986). The differences between the PEF-treatments might have arose from the differences in the non-volatile components in the wines, especially phenolic composition, that can affect the volatility of these compounds (Rodríguez-Bencomo et al., 2011). Apart from (4S,5R)-4-methyl-5-(3-methyl-2-buten-1-yl)dihydro-2(3H)-furanone, control wine and wine from PEF2-treated grapes increased one other volatile furan compound, namely furfural and ethyl 2-furancarboxylate, respectively. Along with Maillard reaction and caramelisation, the formation and increase in furan compounds in stored wines from untreated and low-intensity-PEF-treated grapes (PEF2) can be due to their increase susceptibility towards oxidation (Balboa-Lagunero, Arroyo, Cabellos, & Aznar, 2011; Escudero, Asensio, Cacho, & Ferreira, 2002) or presence of more precursors available in wines from the PEF-treated grapes (Rapp & Mandery, 1986).

5.4 Conclusion

Multiplatform analytical approach combining phenolic profiling, volatile fingerprinting, colour and oenological measurements, and strategic analysis of these data through MVDA techniques, namely PCA and PLS-R, were proven to be a robust method to comprehensively visualise and identify important changes in PEF wines and control wines during bottle storage at 4°C, 25°C, and 45°C. As a function of storage time, phenolic acids increased and monomeric anthocyanins decreased along with the dulling of the wine colour. These changes were accelerated in higher temperatures. At 4°C, a number of esters and acetates was shown to significantly decrease, except in PEF2 wine. At high temperature of 45°C for wine storage, extreme reactions include the increase in the piceid, syringic acid, furan compound, and linalool 3,7-oxide concentrations. These compounds were products of phenolic and aroma compound degradations induced by heat, such as Maillard reaction producing the furan compound and degradation of linalool to produce linalool 3,7-oxide. The observed decrease in caftaric acid and rutin seem to be a result of accelerated hydrolysis reactions and, possibly, copigmentation due to storage high temperature. On the other hand, PEF pre-treatment which produced wines of different phenolic and volatile composition has also affected the evolution of these compounds during prolonged storage. Some of the prominent trends observed in stored PEF wines include significant decrease of anthocyanins in stored wines

from PEF8-treated grapes at 4°C, increase of phenolic acids in PEF wines stored at 25°C, and at 45°C, decrease of citronellol in control and high specific energy PEF (PEF5 and PEF8) stored wines, decrease of 2-phenylethyl acetate in control and low specific energy PEF (PEF2 and PEF6) stored wines, and the formation of more furan compounds in control and low intensity PEF (PEF2) stored wine. These variations, however, are only identified through the list of discriminant markers of each wine. Hence, there is a need for further analysis to appropriately compare the differences between PEF pre-treatments in the storage and temperature stability of the wines. These information aid in systematic selection of compounds sensitive to storage time and temperature, which can be useful in determining wine storage and temperature stability through kinetic modelling. Therefore, the discriminant markers identified in this study can be a reliable representative in differentiating the storage and temperature stability of PEF wines in future studies.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

6.1 General discussion

The application of pulsed electric fields in winemaking especially as a pre-maceration treatment of grape must has been explored. Previous studies have focused on the impact of PEF on the targeted attributes of grape juice or wine, most commonly phenolic compounds, colour, and antioxidant activity. These attributes are commonly measured after PEF treatment or after maceration – alcoholic fermentation (MAF) only, overlooking the influence of malolactic fermentation (MLF) and the maturation and stability of the wine during storage which are essential part of the conventional red winemaking. So far, there is limited literature in studying the effect of PEF on winemaking in a more integrated way based on untargeted and targeted attributes.

The current study has successfully addressed these research gaps. The effect of four different PEF conditions on the winemaking was thoroughly investigated at a commercial scale. This project used Merlot grapes as a case study and a comprehensive multiplatform approach integrating more than a hundred sample attributes including volatile fingerprint, phenolic profile, colour, and oenological properties. These data were processed and analysed using multivariate data analyses, producing effective visualisation of the evolution of sample and of the differences between the samples. Attributes significantly changed by winemaking and storage or impacted by PEF treatment were identified and linked to chemical reactions. The effect of PEF pre-treatment on grapes were followed through different winemaking stages (i.e. pre-maceration (PM), maceration – alcoholic fermentation (MAF), and malolactic fermentation (MLF)) and storage at different temperatures.

In *Chapter 3*, the use of MVDA techniques, particularly principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA), in analysing consolidated phenolic profiles, volatile fingerprints, colour and oenological properties to ultimately select discriminant markers through calculation of variable identification coefficient (VID) have been proven successful in differentiating grape juice at pre-maceration (PM) from the fermenting juice after maceration – alcoholic fermentation (MAF) and from the finished wines after malolactic fermentation (MLF). The major reactions occurring during each winemaking stage represented by the discriminant markers were identified. The grape juice after PM was characterised by the highest amount of sugars (TSS), light colour indicated by

high *L**, acetic acid, octen-3-ol, and C6-compounds, the major green odourants in grape juices. Through vinification, these attributes declined. During MAF, more phenolic compounds and aroma precursors were extracted and transformed. This stage was characterised by higher amounts of phenolic and volatile compounds originating from the grape berries or derivatives of other metabolites extracted such as anthocyanins, flavonols, stilbenes, hydroxycinnamic acids, hydroxybenzoic acids, esters, terpene, alcohol, and ketone. As a result, titratable acidity (TA) increased and colour developed indicated by higher colour intensity (CI). After MLF, the wine contained more hydroxybenzoic acids, hydroxycinnamic acids, esters, and alcohols that differed from the markers in MAF. High concentrations of different flavonol, ketone, and terpene, and the proliferation of volatile fatty acids and norisoprenoid were also an aspect of this stage, where most of compound formation and increase are a product of lactic acid bacteria metabolism.

The same MVDA techniques, type of consolidated data, and marker selection procedure used in *Chapter 3* were performed in *Chapter 4* to identify the main effects of varying PEF conditions such as electric field strength and specific energy applied on the grapes. Comparison of the effects of different PEF conditions immediately after treatment (grape juice), after MAF (fermenting juice), and after MLF (finished wines) was accomplished. As demonstrated in commercial scale processing, (bio) chemical reactions during winemaking (maceration and fermentations) predominate over the effect of PEF treatment of grapes. At PM, PEF treatment on grapes boosted the release of phenolic compounds and minimised the production of 2-hexenal (a volatile compound belonging to the C6 compounds particularly abundant at pre-maceration stage). The extent of these effects depended on the specific energy used during the treatment. The highest extraction of skin- and seed-bound phenolics such as anthocyanins, flavonols, stilbenes, hydroxycinnamic acids, flavanonol, and flavanols was achieved by PEF applications at higher specific energy (47.25 – 49.40 kJ/L), seconded by PEF applications at lower specific energy (16.47 - 18.90 kJ/L). MAF significantly altered phenolic and volatile compositions that the distinction between fermenting juices from grapes PEF treated at low and high specific energies became unclear. Nonetheless, PEF-pre-treated-fermented musts contained more anthocyanins, stilbenes, flavanols, volatile esters, and lower green odourant 1-hexanol compared to the control sample. Interestingly, the differentiation of samples based on the specific energy of PEF applied resurfaced after MLF in finished wines. PEF treatment at higher specific energies at electric field strengths of 33.1 – 41.5 kJ/L produced wines with higher levels of anthocyanins, stilbenes, flavonols,

flavanols, hydroxycinnamic acids, and hydroxybenzoic acids. Between the two PEF conditions of comparable high specific energy, the treatment performed at higher electric field strength produced the most distinct wine.

Through the MVDA techniques PCA and partial least square regression (PLS-R) and discriminant marker selection (using VID) from the volatile fingerprints, phenolic profile, colour, oenological properties of stored wine samples, important changes driving the stability of PEF wines and control (no PEF) wine during storage at 4°C, 25°C, and 45°C were identified in *Chapter 5*. The impact of storage time, temperature, and PEF pre-treatment were elucidated in this chapter. As a function of storage time, the concentration of hydroxycinnamic acids and hydroxybenzoic acids increased while monomeric anthocyanins decreased resulting in duller colour at the end of storage. Storage temperature affected the rate and the type of reactions occurring during storage. The most prominent effect of storage at 4°C for 180 days was the loss of esters and acetates. Meanwhile, storage at 45°C for a shorter period of 45 days accelerated reactions altering both phenolic and volatile compositions of the wines. These reactions include (*i*) the speculated re-glycosylation of resveratrol or isomerisation reactions increasing piceid concentrations; (*ii*) anthocyanin cleavage producing more syringic acid; (*iii*) caramelisation and Maillard reaction forming furan compounds; (*iv*) degradation of terpene alcohol linalool producing the terpene oxide linalool 3,7-oxide; (*v*) hydrolysis of the tartaric ester caftaric acid into corresponding free acid caffeic acid; and (*vi*) copigmentation with anthocyanins or degradation into the aglycone quercetin resulting to the decrease of the flavonol glycoside rutin. The effect of PEF conditions such as electric field strength and specific energy applied on grapes was carried over onto the wines as far as after storage. Wine made from grapes treated at the highest electric field strength and energy (PEF 8) deemed to be the most distinct wine due to high concentrations of anthocyanins and other phenolic compounds. This wine is the only wine with significant decrease in anthocyanins after storage at 4°C for 180 days. After storage at 25°C for 120 days, only wines made from PEF treated grapes had increased hydroxycinnamic acids and hydroxybenzoic acids which might be due to increased levels of precursors – tartaric esters and anthocyanins, respectively. Storage at elevated temperature of 45°C for 56 days showed varying effect on important volatile compounds, which tended to be a similar compound in wines from grapes PEF-treated at comparable specific energy. After storage, wines made from grapes treated at high specific energy PEF (PEF5: 47.25 kJ/L and PEF8: 49.40 kJ/L) had significant loss of citronellol, while those treated at low

specific energy PEF (PEF2: 18.90 kJ/L and PEF6: 16.47 kJ/L) had a reduced concentration of 2-phenylethyl acetate. Control wine had decreased concentrations of both volatile compounds. Moreover, control wine and wine made from grapes treated at low electric field strength of 33.1 kV/cm and low specific energy (PEF2) formed significant amounts of one more furan compound in addition to one common in all wines.

This study clearly showed that multiplatform analyses coupled with chemometric technique could be used as a robust technique to gain a more integrated assessment on various complex changes taking place during fermentation. These findings also show the potential of using this technique for optimising PEF processing parameters (e.g. electric field strength and specific energy) to produce red wine with unique composition.

6.2 Final conclusion

This study has successfully demonstrated the feasibility of integrating targeted profiling of phenolic compounds, colour, and oenological properties and untargeted volatile fingerprinting coupled with multivariate data analyses and discriminant marker selection in investigating grape juice, fermenting juice, and wine matrices. The evolution of phenolic and volatile composition and other properties during winemaking and storage has been essentially visualised in bi-plots and encapsulated into the important discriminant markers representing major chemical reactions crucial to the transformation of grapes to wine and to wine stability. Furthermore, this technique has elucidated the effect of PEF treatment at different stages of winemaking and storage. Hence, the technique can be used as a toolbox in optimising winemaking processes and in optimising PEF parameters.

Several reactions in conventional winemaking of Merlot red wine have been highlighted including the decline in C6 compounds after PM, the extraction of phenolic compounds and formation of fermentation aroma during MAF, and transformation of the extracted phenolic compounds into their derivatives and further evolution of the aroma after MLF. During storage, the increase in phenolic acids, decrease in anthocyanins and dulling of colour was predominant. However, at elevated temperature, the volatile composition of wine was greatly modified.

Pre-treating the grape must with PEF, on the other hand, has been demonstrated to impact the extraction of phenolic and aroma precursors. Consistently, juice and wine samples from

grapes PEF-treated at high specific energies (47.25 - 49.40 kJ/L) were differentiated from those PEF-treated at lower specific energies (16.47 - 18.90 kJ/L). All PEF treatments were also shown to be significantly different from the control. These findings imply the potential of employing combined profiling, fingerprinting, and chemometrics in optimising PEF treatment to create novel wines with improved phenolic and volatile composition expressed as improved colour, aroma, taste, and health effects.

6.3 Limitations and future research

The findings in this study have to be seen in light of some limitations. First, optimum maceration time for the PEF-treated grape must was not determined considering the increased extraction of phenolic compounds and aroma precursors at a shorter period of time due to the PEF treatment. During winemaking, PEF-treated and untreated grape must were macerated for the same number of days. For future studies, optimum maceration time can be determined and carried out for both PEF-processed and unprocessed grapes, whose products can be further chracterised and compared. Second, phenolic and volatile compounds dictate the colour, taste, and aroma of the wines. Hence, considering the evidenced effect of different PEF treatments on the identified phenolic and volatile markers in the finished wine, sensory evaluation of these wines is required to be able to further describe the impact of these treatments on the overall quality of wine. Third, the study on the stability of the wines during storage at varying temperatures has directed the focus on phenolic and volatile markers which may be both time and temperature sensitive. In this regard, kinetic modelling on the evolution of these markers during storage can help establish quality indicators and thermal stability of wines made from PEF treated grapes.

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